The Airway Microbiota: Association with Inflammatory Patterns in Chronic Rhinosinusitis

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by
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List of publication

List of publications

This thesis is based on the following articles submitted to, accepted, or published in international peer reviewed journals.

1. Thanit Chalermwatanachai, Leydi Carolina Velásquez, Claus Bachert. The microbiome of the upper airways: focus on chronic rhinosinusitis. The World Allergy Organization Journal 2015;8(1):3.

2. Thanit Chalermwatanachai, Nan Zhang, Gabriele Holtappels, Claus Bachert. Association of mucosal organisms with patterns of inflammation in chronic rhinosinusitis. PLoS One 2015;10(8): e0136068.

3. Thanit Chalermwatanachai, Ramiro Vilchez-Vargas, Gabriele Holtappels, Tim Lacoere, Ruy Jáuregui, Frederiek-Maarten Kerckhof, Dietmar H. Pieper, Tom Van de Wiele, Mario Vaneechoutte, Thibaut Van Zele, Claus Bachert. Chronic rhinosinusitis with nasal polyps is characterized by dysbacteriosis of the nasal microbiota (Revised version was submitted to Scientific Reports on 24th January 2018)

Preface

Preface

Chronic rhinosinusitis is characterized by substantially different inflammatory profiles, so called endotypes. Traditionally, studies on the association of specific bacterial patterns with inflammatory profiles of diseases had been dependent on clinical phenotypes and bacterial culture. Since the 1980s, molecular biology methods have allowed us to study microbial communities by detecting the genetic material of microbes in a given environment. Molecular techniques have discovered un-cultivable microbes and identified complex microbial communities. The microbiota is much more diverse than previously recognized including those found in the upper airways. It is widely believed that microbiota is important for maintaining human health. An alteration of microbiota may contribute to the inflammatory disorders and thus associated with inflammatory endotypes, further differentiating clinical phenotypes.

The common hallmark of chronic rhinosinusitis pathophysiology is an inflammation. A key feature of any microorganism is its ability to activate the host's immune system. Microbiota play a critical role in the induction, training, and function of the host's immune system. Therefore, the study of the pathophysiology of chronic rhinosinusitis is necessary to establish the relationship between the microbiota and inflammatory patterns. Such investigations may elucidate the path to therapeutic approaches in correcting an imbalanced microbiota.

In the introductory chapters, state of the art is presented. First, a brief overview of chronic rhinosinusitis pheno- and endotypes will be given. In the following part, the hypothesis of the pathogenesis of chronic rhinosinusitis will be presented, and the microorganisms in chronic rhinosinusitis are presented. Lastly, the microbial ecology in chronic rhinosinusitis is reviewed.

The first part of studies in this thesis will focus on the association between intra-mucosal microorganisms and inflammatory patterns. In a second part, the composition and diversity of sinonasal microbiota on mucosal surface are determined. In the closing chapter, the impact of our findings will be presented.

List of abbreviation

List of abbreviations

ARS	: Acute rhinosinusitis
CF	: Cystic fibrosis
CRS	: Chronic rhinosinusitis
CRSsNP	: Chronic rhinosinusitis without nasal polyps
CRSwNP	: Chronic rhinosinusitis with nasal polyps
CRSwNP-A	: Chronic rhinosinusitis with nasal polyps without co-morbid asthma
CRSwNP+A	: Chronic rhinosinusitis with nasal polyps with co-morbid asthma
DNA	: Deoxyribonucleic acid
ECP	: Eosinophil cationic protein
FISH	: Fluorescence in situ hybridization
IFNγ	: Interferon gamma
Ig	: Immunoglobulin
IL	: Interleukin
MPO	: Myeloperoxidase
NP	: Nasal polyps
OTUs	: Operational taxonomic unit
PCR	: Polymerase chain reaction
Phy	: Phylotype
PNA-FISH	: Peptide nucleic acid fluorescence in situ hybridization
RNA	: Ribonucleic acid
rRNA	: Ribosomal ribonucleic acid
SE-IgE	: Specific IgE to staphylococcal enterotoxins
TNFα	: Tumor necrosis factor alpha

Chapter 1.
Chronic rhinosinusitis:
From phenotypes to endotypes

Chapter 1. Chronic rhinosinusitis: From phenotypes to endotypes

Definition of rhinitis and rhinosinusitis

Rhinitis and sinusitis are inflammatory diseases of the mucosa of the nasal and paranasal cavities. The cardinal symptoms of rhinitis, described by the ARIA guidelines, are anterior and posterior rhinorrhea, sneezing, nasal blockage, and/or nose itching [1]. The combined term "Rhinosinusitis" was invented in 1997 by a Task Force of the Rhinology and Paranasal Sinus Committee. Sinusitis rarely occurs in the absence of rhinitis, and the nose and sinuses are contiguous structures sharing vascular, neuronal, and interconnecting anatomic pathways [2]. Moreover, the inflammatory mediator profile in the nasal mucosa of patients imitates the profile found in sinus mucosa [3]. As proposed by the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) expert committee, rhinosinusitis is defined as inflammation of the nose and the paranasal sinuses characterized by two or more symptoms, one of which should be either nasal blockage/obstruction or nasal discharge (anterior/posterior nasal drip). Other symptoms can be facial pain/pressure, reduction or loss of smell, or both [4]. Acute rhinosinusitis (ARS) is clinically defined as symptoms lasting less than 12 weeks with complete resolution. Chronic rhinosinusitis (CRS) is defined as symptoms on most days lasting at least 12 weeks without complete resolution. The prevalence of physician-diagnosed CRS ranges from approximately 1% to 9% of the general population [5]. In 2011, a large-scale adult population study showed the prevalence of CRS to be 10.9% in Europe [6]. CRS is a chronic malady with a considerable economic burden resulting from the costs of diagnostic tests, medical and surgical therapies, lost and reduced school and work productivity, and detrimental impacts on physical and emotional health.

Classification of chronic rhinosinusitis

CRS is usually further categorized based on the absence or presence of nasal polyps (CRS without nasal polyps, CRSsNP; or CRS with nasal polyps, CRSwNP). Although both are characterized by mucopurulent drainage and nasal

obstruction, CRSsNP is frequently associated with facial pain/pressure/fullness whereas CRSwNP is frequently characterized by hyposmia [7]. Based on histopathology, CRSsNP is characterized by fibrosis, basement membrane thickening, goblet cell hyperplasia, subepithelial edema, and mononuclear cell infiltration, whereas CRSwNP is characterized by an intense edematous stroma with albumin deposition, formation of pseudocysts, and subepithelial and perivascular inflammatory cell infiltration [8]. Nasal polyps (NP) are believed to arise in the nasal mucosa as a result of chronic inflammation. NP is defined as pedunculated lesions as opposed to cobblestoned mucosa, endoscopically visualised in middle meatus. CRSwNP, a clinical phenotype, is found in up to 4% of the population [9]. Although, the clinical presentations of CRS are useful in treatment planning, clinically defined phenotypes do not provide full insight into the potential underlying cellular and molecular mechanisms of CRS [10]. CRS is a complex disease with several variants caused by different cellular and molecular mechanisms [11]. The characterization of this heterogeneity supports the concept that CRS consists of multiple biological subtypes, or endotypes, which are defined by distinct pathophysiologic mechanisms that might be identified by corresponding biomarkers [10]. Typically, patterns of cytokines for CRSsNP are T helper 1(Th1)-driven, whereas CRS with eosinophilic NP is T helper 2(Th2)-biased and CRS with neutrophilic NP expresses increased amounts of Interferon gamma (IFNγ) and/or Interleukin (IL)-17 or none of those. Sinonasal tissues from cystic fibrosis patients demonstrate high concentrations of IL-17 and IL-8 [12]. Existing evidence suggests an individual inflammatory pattern for patients with CRS. Recently CRS has been classified into ten groups based on cluster analysis of biomarkers (Fig 1)[13]. From these 10 biological patterns CRS can be further classified into four endotypes based on the pathophysiological and therapeutic principles (Table 1). Those endotypes are defined as: (1) Non-inflammatory endotype, (2) Neutrophil endotype, (3) Mainly eosinophilic endotype (increased IL-5, ECP, IgE, and albumin) and (4) Cleary eosinophilic/SE-IgE endotype (much increased IL-5, IgE, ECP, albumin, and SE-IgE positive) (Table 2)[14]. In the whole field of medicine, recognition of endotypes of chronic inflammatory diseases is becoming more and more important because it is apparent that a traditional management approach of ‘‘one size fits all’’ does not adequately treat many patients whose symptoms remain uncontrolled and who have severe disease.

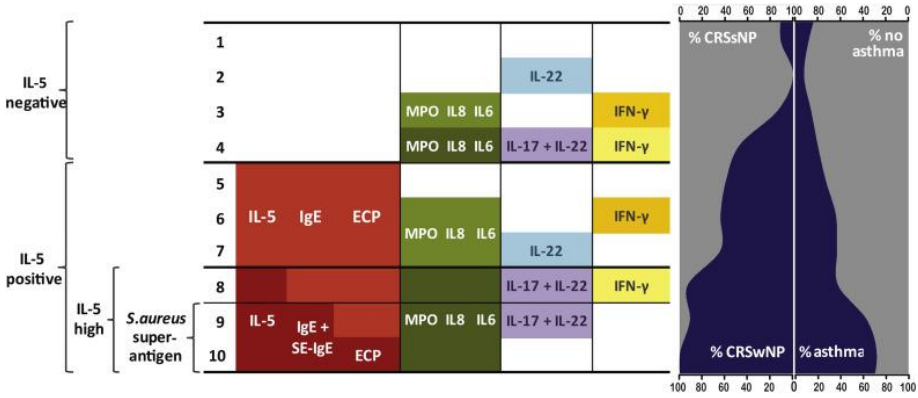

Figure 1. Simplified graphic showed clusters and their characteristic cytokines, as well as the distribution of CRSsNP versus CRSwNP and asthma. For cytokines, white indicates no increased concentration, light colors indicate moderately increased concentrations, and dark colors indicate strongly increased concentrations. Horizontal lines indicate groups of clusters, as determined by IL-5, SE-IgE, and CRSwNP and asthma characteristics. ECP stands for Eosinophil cationic protein, MPO for myeloperoxidase. (Source: Tomassen P, Vandeplas G, Van Zele T, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. The Journal of Allergy and Clinical Immunology. 2016)

Table 1. Characteristics of CRS phenotypes and endotypes.

Phenotypes	CRS without NP (CRSsNP)					CRS with NP (CRSwNP)				
Clinical manifestation	Pain					Hyposmia				
Treatment plan	Medication → Surgery									
Quality of life	Less impacts					High impacts				
Co-morbid asthma	Low					High				
Remodeling	Fibrosis					Edema				
Endotypes	Pauci-inflammatory → Neutrophilic → Eosinophilic									
Cellular mechanisms	?	Th 22	Th1	Th1 Th17	Th1 Th17 Th22	Th 2	↑Th2 Th1	↑Th2 Th17	↑Th2 Th22	↑Th2 super-antigen

Table 2. Endotypes of CRS with clinical findings

Endotype	**Clinic**
Non-inflammatory endotype	90% of CRSsNP, asthma in <20%
Neutrophil endotype	70% of CRSsNP, asthma in <20%
Mainly eosinophilic endotype: increased IL-5*, ECP, IgE and albumin	70% of CRSwNP, asthma in 20-40%
Clearly eosinophilic/SE-IgE endotype: much increased IL-5**, IgE, ECP, and albumin with SE-IgE positivity	>90% of CRSwNP, asthma in >60%

*Increased IL-5 means the level of IL-5 between 100 and 200 pg/mL.

**Much increased IL-5 refers to the level of IL-5 above 200 pg/mL.

(Source: Bachert C, Holtappels G. Pathophysiology of chronic rhinosinusitis, pharmaceutical therapy options. GMS Current Topics in Otorhinolaryngology, Head and Neck Surgery. 2015)

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Chapter 2.
Pathogenesis of Chronic rhinosinusitis: Hypothesis

Chapter 2. Pathogenesis of Chronic rhinosinusitis: Hypothesis

Chronic rhinosinusitis (CRS) is an umbrella term for a heterogeneous group of debilitating chronic inflammatory sinonasal diseases. CRS disease occurs at the site of interface between the host and the environment. A number of hypotheses have been proposed based on the concept that CRS disease results from a dysfunctional interplay between individual host characteristics and factors exogenous to the host.

Etiology of chronic rhinosinusitis

Universal to the pathophysiology of CRS is the persistence of inflammation. A number of hypotheses have been proposed to explain all or part of the clinical CRS spectrum [1]. However, the cause of this inflammation in an individual patient is most often indiscernible. Different immune and inflammatory mechanisms are involved in CRS pathogenesis. Historically, CRSsNP was considered to be the result of an incompletely treated or unresolved bacterial infection, while CRSwNP was regarded as a noninfectious disorder linked to atopy[2]. Research over the last 20 to 30 years has indicated that the etiology and pathogenesis for both forms are far more complex and overlapping [3]. Nowadays, CRS disease is described as an umbrella term for a heterogeneous group of inflammatory conditions of sinonasal mucosa resulting from the interaction of multiple host and environmental factors. Here, we discuss the etiology of CRS as environment-related hypothesis (fungal pathway, microbial elements pathway), host-related hypothesis (eicosanoid pathway, immune pathway), and microbiota dysbiosis pathway.

Fungal pathway

Fungi have been considered as causal pathogens in CRS diseases by researchers from the Mayo Clinic [4]. Evidence of fungi, eosinophilic mucin, and eosinophils in the nose and sinuses is present in nearly all patients with CRS. Our group published data that fungal hyphae, Charcot Leyden crystals and eosinophilic

mucin could be found in 7% of CRS patients and was related to recurrence of disease [5]. However, multiple studies are unable to detect a significant difference in fungal isolation between CRS patients and healthy control subjects [6]. Although fungal microorganisms were identified in the sinonasal cavities of essentially 100 % of both CRS patients and healthy control subjects, CRS cases show an exaggerated immune response to fungal antigens [7]. At present, the fungal hypothesis has been largely dismissed for multiple reasons. The most important reason is that treatment of CRS patients with antifungal agents did not show any evidence of efficacy in modifying the CRS disease process [8]. Currently, fungi are no longer viewed as the principal cause in CRS context. Fungi, bacteria and virus probably contribute to some extend to CRS disease under certain circumstances.

Microbial elements pathway

Early research on CRS pathophysiology was mainly considered on the basis of infection model of disease, with a focus on identifying a pathogenic microbial driver for the inflammatory response. Suggestions of a viral cause of CRS have largely been disappointing because of the lack of convincing evidence for such a cause [9-11]. Prevailing evidence indicated bacteria to be a promising infectious agent that caused CRS. Previous culture-based studies counted *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* as potential pathogens. Since the discovery of IgE antibodies to *S. aureus* enterotoxins A and B in nasal polyp tissue homogenates [12], much of further research in the pathogenesis of CRS has focused on the possible role of *S. aureus* and its products. *S. aureus* colonization rates have been higher in CRSwNP patients than those of healthy control subjects and CRSsNP patients [13]. Furthermore immunoglobulin E specific to staphylococcal enterotoxins (SE-IgE) has been detected in polyp homogenates, but not in control or CRSsNP tissues [12]. The level of SE-IgE in the sinonasal tissue is associated with high total IgE titers [12]. This IgE antibody appears to be functional and may induce mast cell degranulation to numerous inhalant allergens [14]. Moreover *Staphylococcus* enterotoxins (SEs) are regarded as superantigens. SEs, produced by the *S. aureus*, amplifies the local eosinophilic response [12]. SEs may serve as an allergen and may

contribute to persistent inflammation by continuously activating mast cells. SEs significantly down regulates prostaglandin E2, cyclooxygenase 2, and prostaglandin EP2-receptor mRNA expression, pointing to an effect of staphylococcal superantigen on eicosanoid metabolism in the upper airway tissue [15]. Bachert and colleagues have reviewed the role of staphylococcal superantigen in the upper airway [16-18]. Other virulence factors would be e.g. serine protease-like proteins (Spls), inducing a Th2-biased inflammatory response in the airways via an IL-33-dependent manner [19,20]. Current evidence indicates that *S. aureus* and its secreted proteins are disease modifiers for the development of nasal polyposis, rather than etiologic agents [16]. There is only a little evidence so far that *S. aureus* can cause disease in the human in an otherwise disease-free airway. Other microbes also may play a part in the pathomechanism of CRS. Today, it is generally accepted that the "singular cause vision" is an inadequate approach to explain the pathophysiology of CRS.

Eicosanoid pathway

Eicosanoids are signaling lipids that regulate pivotal homeostatic functions such as inflammation, immunity, and messenger networks in the central nervous system [21]. Their synthesis starts when the cell, activated by mechanical trauma, cytokines, pathogens or other stimuli, triggers the release of membrane-bound fatty acids that are then metabolized via the cyclooxygenase and/or the lipoxygenase pathways. Alterations in the arachidonic acid pathway, leading to an imbalanced production of eicosanoids, have been linked to the pathophysiology of CRSwNP diseases, asthma, and aspirin-exacerbated respiratory disease [22]. This imbalance is characterized by increased synthesis of pro-inflammatory mediators such as cysteinyl leukotrienes by inflamed tissue in addition to decreased anti-inflammatory molecules such as prostaglandin E2 release when compared with normal tissue, correlating with the inflammatory pattern and severity of the diseases [22]. Furthermore, anti-leukotriene agents improved subjective and objective symptoms of CRSwNP patients, especially patients with comorbid asthma [23,24]. Leukotriene inhibitors reduced nasal polyp size and decreased recurrence rate of nasal polyp

after surgery [25]. These evidences suggest that eicosanoids could contribute to the pathophysiology of CRS.

Immune dysfunction pathway

Due to a failure of defining microbial agents, e.g. fungi and *S. aureus,* as the primary cause of CRS, the immune dysfunction hypothesis was proposed to fulfill the pathomechanism of CRS. The possible mechanisms are deficiency of innate immune response, and aberrant communication and/or signaling between the innate and adaptive immune responses. With regard to the tissue level, macrophages in NP tissues showed decreased engulfment of *S. aureus* [26]. Furthermore, nasal tissues from CRSwNP patients showed a significant deficit in IFN-γ response to viral infection [27]. These defects may increase pathogen access and result in an aberrant immune response. Peripheral blood leukocytes isolated from CRS patients produce IL-5 when challenged by common microbial agents [28]. Moreover, there is a meta-analysis study that reveals an immunoglobulin deficiency in CRS patients [29]. Approximately one in four patients with difficult-to-treat CRS had IgG, IgA, and IgM deficiencies [29]. These findings indicate an abnormal immune system in CRS patients, although we cannot proof if this aberrant immune system is a cause or a consequence of inflammation. Additional studies on the immune system in CRS patients are necessary to make a conclusion.

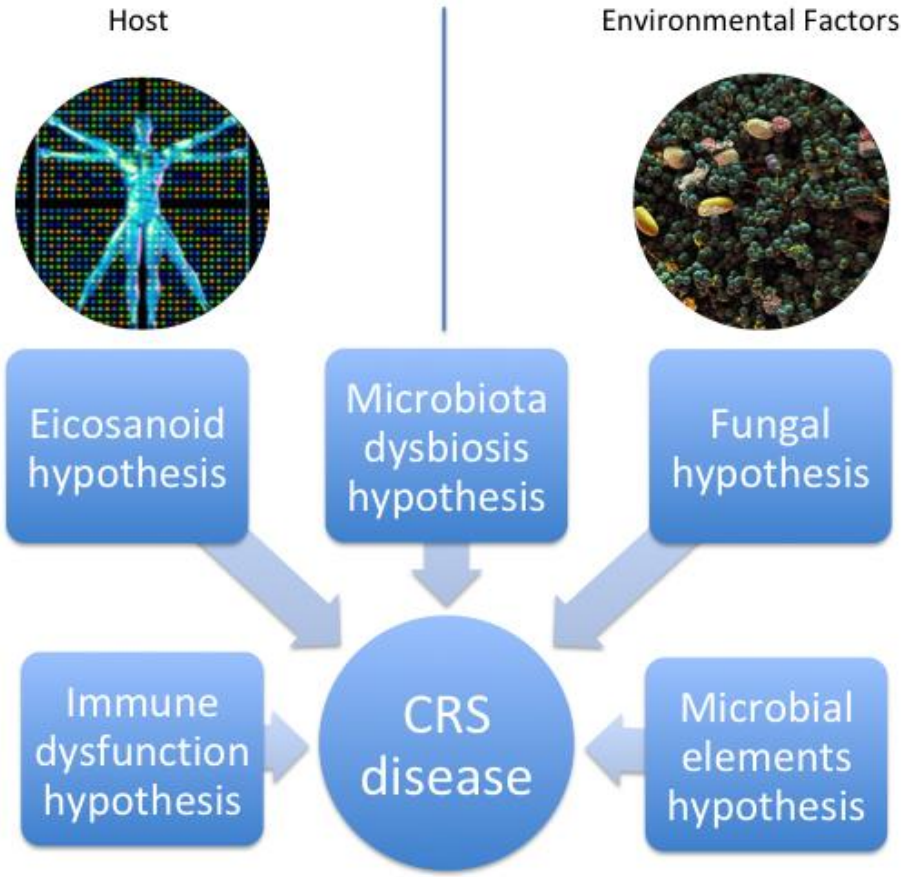
Microbiota dysbiosis pathway

There is a general consensus that the microbiota have implications in human health and disease [30]. The role of microbiota in shaping the immune response has been largely recognized. Stable microbiota contribute to fortification of the epithelial barrier and development of innate and adaptive immune properties [31]. This hypothesis proposes that changes in host-microbiota relationship (dysbiosis) can result in an aberrant immune response leading to chronic inflammation. Two possible mechanisms into the pathogenesis of CRS are (1) colonization resistance, and (2) tonic activation of immune response. The microbiota dysbiosis hypothesis incorporates host and environmental factors. It is the broadest and most inclusive

hypothesis for CRS etiology and pathogenesis. This hypothesis can account for, rather than conflict with, the most prominent data supporting the other postulations. Studies about the impact of sinonasal microbiota in the pathogenesis of CRS are beginning to emerge. Prevailing studies report that the characteristics of sinus microbiota in CRS cases were similar to those of healthy control subjects [28,32,33] whereas other analyses, making use of CRS phenotypes or endotypes, demonstrated different compositions of resident bacterial communities [34-36]. The amount of evidence is still relatively scant; hence, sinonasal microbiota will require more extensive studies to address the role of microbiota dysbiosis in the pathogenesis of CRS.

Conclusions

There are many different hypotheses related to CRS etiology. Fungi and bacteria have been implicated as important environmental factors contributing to chronic mucosal inflammation and have formed the basis for discussion of the fungal pathway and microbial elements pathway. In contrast, the eicosanoid pathway and the immune dysfunction pathway highlight the contribution of host variables. Host and environmental factors are entwined by the microbiota dysbiosis pathway (Fig 1). There is strong evidence supporting an important role for both host and environmental factors in CRS pathophysiology. We have an incomplete understanding of the molecular pathways that lead to the tissue manifestations and clinical symptomatology defining CRS. Therefore, identification of specific disease subgroups within the broad concept of CRS, linked to etiology is an important and challenging task in future research.



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Chapter 3.
Microorganisms in Chronic rhinosinusitis

Chapter 3. Microorganisms in Chronic rhinosinusitis

An infectious etiology for CRS has long been postulated [1]. There is significant scientific evidence that acute rhinosinusitis (ARS) is primarily an episodic viral infection of the paranasal sinuses. However, current evidence is relatively scant for supporting viruses as a significant component in the pathogenesis of CRS. According to studies using PCR techniques, there was no significant difference in the rate of viral infections between healthy control subjects and CRS cases [2]. Furthermore, viral elements have not been detected in sinonasal tissues of CRS patients [3]. The most important progression of acute rhinosinusitis is superinfection of bacteria on mucosa damaged by viral infection. The most common pathogens associated with bacterial acute rhinosinusitis are *Streptococcus pneumoniae* and *Haemophilus influenzae* [4]. The transition of bacteria from acute to chronic status has been evaluated by repeated endoscopic sinus aspirations [5]. Initially, *S. pneumoniae, H. influenzae* and *Moraxella catarrhalis* were recovered. Failure to respond to therapy was associated with the appearance of resistant bacteria in subsequent aspirates. These included *Fusobacterium nucleatum* and species of the genera *Prevotella*, *Porphyromonas* and *Peptostreptococcus*. Based on conventional culture techniques, there are significant differences in the microbiology of CRS as compared with ARS [6]. *Staphylococcus aureus*, *Staphylococcus epidermidis*, and anaerobic Gram- negative bacteria predominate in CRS patients. Recovery of Gram-negative rods, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter* spp., and *Escherichia coli*, were also reported [7]. These bacteria are rarely recovered from middle meatus cultures obtained from healthy individuals. Their isolation from symptomatic patients suggests their pathogenic role [8]. These data provide evidence that bacteriology in CRS patients is different from that of healthy control subjects before acquisition of CRS disease. It is fair to say that bacteria are encountered frequently in the setting of CRS; the microbial community of the paranasal sinuses seems to be altered. This bacterial community is often polymicrobial and may exhibit significant antibiotic resistance.

We reported for the first time that an increased rate of *S. aureus* colonization in middle meatus nasal swabs from CRSwNP patients (63.7%) was higher than those of CRSsNP patients (27.3%). Even higher rates were detected in CRSwNP patients with asthma (66.7%) and aspirin hypersensitivity (87.5%), whereas the *S. aureus* colonization rate between CRSsNP patients and healthy control subjects did not reach a statistically significant difference [9]. This evidence highlighted the role of *S. aureus* in pathogenesis of CRS disease. The colonization rates of *S. aureus* in CRSwNP patients are positively correlated with *S. aureus* enterotoxin-specific IgE (SE-IgE) in nasal tissue homogenates; however, *S. aureus* colonization rates always exceeded the SE-IgE rates. The presence of SE-IgE in nasal polyps is strongly associated with co-morbid asthma. In a subgroup of CRSwNP patients, *S. aureus* seemed to enhance type 2 inflammation. *S. aureus* may initiate and maintain chronic inflammation via its various secreted proteins, although various other microbes may play a role either in the progression or the termination and prevention of sinus disease.

Most microbes cannot be cultured in the laboratory. It is estimated that culture-dependent methods demonstrate only 1% of microorganisms in nature [10]. Since the application of molecular methods, DNA-based techniques have been developed allowing researchers to identify bacteria in sample material without cultivation [11]. The study of the genetic material of microorganisms in a particular environment, termed microbiota, is one of the new perspectives in human health research [12], including CRS. An emerging body of evidence in the field of human microbial ecology is redefining our perception of the human superorganism and the delicate balance between the host immune response and the microbial populations [13]. The Human Microbiome is the collection of all the microorganisms living in association with the human body. The traditional hypothesis was that humans live with ten times more bacterial cells than human cells [14], however, more recent estimates have lowered that to 3:1 and even to approximately the same number [15]. These microbes are generally not harmful to human, in fact they are essential for maintaining health. Microbiologists point out that if all of a person's DNA were mixed with the body's entire bacterial DNA, that person would be genetically more

bacterial than human. Bacterial gene content is more than 100 times that of the human genome [16]. Cooperative interactions between microbes and their hosts typically involve microbial participation in host functions such as defense, metabolism, and reproduction. For example, they produce some vitamins, break down our food to extract nutrients we need to survive, teach our immune systems how to recognize dangerous invaders and even produce helpful anti-inflammatory compounds that fight off other disease-causing microbes.

Many microorganisms colonize humans. Populations of microbes inhabit the skin and mucosal surfaces in various parts of the body [17]. Traditional microbiology has focused on the study of individual species as isolated units. Historically, research on pathogenesis of the condition neglected the roles of resident microbial communities (where most pathogens function). Indeed, many researchers eliminate the native microbial communities of their model hosts to simplify analysis of pathogens that infect them. Because interest in the involvement of those communities in host health centers largely on how they modulate host defenses. More efforts are needed to address the purely microbial interactions that influence how pathogens affect those microbial communities as they also colonize their hosts. It is clear that chronic inflammatory diseases represent a significantly more complex interaction between microbial communities and host inflammatory response than was previously understood. Studies of a variety of microorganisms including eukaryotes (fungus), archaea, bacteria and viruses should pave the way for more understanding of how the host microbiome contributes to pathogenesis. In the context of CRS, roles of bacteria in the pathogenesis are currently being reassessed. Modest improvement in some patients with CRS can occur with antibiotic therapy [18]. So far there is no convincing evidence to support viruses as a cause of CRS [2,3,19]. The potential roles of fungi are questioned by the failure of antifungal therapeutics to improve CRS symptoms [20].

The role of the microbiota in regulation of epithelial and airway inflammation has seen a recent explosion in promising research. Many investigators have reported that the quality of sinus microbiota in CRS is similar to that of control groups, but several studies indicate that alterations in the airway microbiota can be associated with inflammatory processes. The nasal microbiota appear to have potentially promising roles in many aspects of sinus health and disease. The research into the consequences of sinus dysbiosis can thus be used to explore and further define mechanisms of disease. The study with well-defined subgroups will be able to clearly demonstrate different pathologic or immunologic characteristics. Exploration of the relationships between microbes and their impact on the sinonasal immune responses may lead to a greater understanding of the pathogenesis of CRS and thus result in new strategies for its treatment.

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Chapter 4.
Microbial Ecology in Chronic rhinosinusitis

Chapter 4. Microbial Ecology in Chronic rhinosinusitis

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Chronic rhinosinusitis (CRS) is a persisting inflammatory condition of nasal and paranasal cavities. Microorganisms are believed to be important contributors to the inflammatory response seen in these patients. Traditionally, studies on the association of bacterial community and CRS disease depended on bacterial culturing techniques. The advent of molecular biology methods has allowed us to study microbial communities and demonstrated that the human being inhabited by abundant and diverse bacteria that cannot be detected by culture-based methods. Molecular techniques have identified complex microbial communities. Parallelling groundbreaking research in the gastrointestinal tract suggests that the gut microbiota may contribute to chronic inflammatory disorders such as inflammatory bowel disease and atopy. Following this perspective, it is likely that the respiratory microbiota may also have an impact on development and severity of airway inflammation.

Techniques in microbial community studies

Historically, Gram stain and microscopy identify *in situ* the members of a microbial community. The staining properties and morphology methods could distinguish many broad clades of bacteria but were non-specific at lower taxonomic levels. Nowadays, there are two principal approaches to analyze the microbial community in a given environment: culture-dependent and culture-independent techniques.

Culture-dependent methods involve isolation and culturing of microorganisms prior to their identification according to morphological, biochemical or genetic characteristics. These methods are time-consuming, and biased, as certain media and growth conditions favor the growth of some bacteria

over others. In addition, this approach may not provide a true reflection of the diversity of microbes in a sample. A “no growth” result does not necessarily imply that a sample is sterile. It is estimated that up to 99% of microorganisms observable in nature typically are not cultivated using standard techniques [1]. Un-cultivability is a wide-spread condition that includes: (i) organisms for which the specific growth requirements (nutritional, temperature, aeration, etc.) are not fulfilled; (ii) slow-growing organisms, out-competed in the presence of fast-growing microorganisms and (iii) disfavored organisms, which cannot stand the stressful conditions imposed by cultivation [1]. This approach camouflages the true bacterial community in a sample. Thus, there needed to be a better approach to analyze these microorganisms.

Since the 1980s, the application of molecular detection methods has allowed culture-independent investigations of the microbial communities [2]. Culture-independent methods explore microorganisms in a sample by detecting the basic unit of life, nucleic acids. The earliest DNA-based methods examine the community by hybriding genes of interest (i.e., fluorescence in situ hybridization: FISH and Microarray) or amplifing specifically-targeted genes (such as denaturing/temperature gradient gel electrophoresis: DGGE/TGGE, restriction fragment length polymorphisms: RFLP). Table 1 summarizes molecular methods of microbial detection. Molecular techniques have proven effective in characterizing complex microbial assemblages in environmental samples [3]; however, an important caveat of molecular techniques is the ability to detect the genetic material of non-viable microorganisms. Fundamentally, a microbial community consists of a collection of individual microbe cells, each carrying a distinct complement of genomic DNA. As a full sequence of every genome in every cell is impractical, microbial ecology has defined a number of genes or other DNA sequences that can assign microbes to a particular taxon. There are different DNA sequences, which can be chosen for analyzing different populations including ribosomal protein subunits, elongation factors, and RNA polymerase subunits. Of those, the 16S ribosomal RNA (16s rRNA) gene is widely applied for exploration of bacterial diversity in the environment because of (i) its presence in all bacteria, often existing as a multigene family, or operons; (ii) the stability of the 16S rRNA gene, suggesting that random sequence changes are a measure of time (evolution) rather than a reflection of different microbiota; and (iii) the size of 16S rRNA genes (1,500 bp) being large enough for informatics purposes [3].

Table 1. Molecular methods of microbial detection

Technique	Description
FISH DNA probe	Fluorescently labeled specific oligonucleotide probes are hybridized to a microbial community for marker genes.
Microarrays	Fluorescently labeled nucleic acids are hybridized to organism-specific oligonucleotides placed onto silicon plates and visualized by using fluorometry.
PhyloChip	The PhyloChip assay is a microarray-based method that identifies and measures the relative abundance of microbial taxa. This approach relies on the analysis of the 16S ribosomal RNA gene sequence.
Ibis T5000 biosensor	A nucleic acid with a target sequence is amplified by PCR then products are injected to high-performance electrospray ionization mass spectrometry and base-composition analysis.
DGGE/TGGE	DNA fragments from a sample are amplified by PCR; products are then subject to gel electrophoresis and increasing either concentration of denaturing reagents or temperature; unique dsDNA fragments will then migrate and melt at different positions along the gel.
RFLP	DNA sample is digested by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.
Quantitative PCR	Primers and fluorescent labels are used to quantify the abundance of select DNA templates.
Sequencing	Genes of conserved loci with hypervariable regions (i.e., 16S rRNA) are amplified, sequenced, and compared with databanks of known gene sequences for taxa identification.

Moreover, there are several available databases of 16S rRNA gene with reference sequences and taxonomies such as Greengenes [4], SILVA [5] and the Ribosomal database project [6].

The introduction of next generation sequencing (NGS) in 2005 [7] changed the history of genomic research as it increased sequencing throughput (Second-generation DNA sequencing), and did not require prior cloning steps (Third-generation DNA sequencing) [8]. NGS can be used to sequence every nucleotide in an individual's DNA, or can be limited to smaller portions of the genome. Second-generation DNA sequencing platforms are commercially available allowing us to sequence DNA much more quickly and cheaply. Third-generation DNA sequencing uses single DNA molecules rather than amplified DNA as a template. Thus, third-generation DNA sequencing potentially eliminates errors in DNA sequence introduced in the laboratory during the DNA amplification process. NGS technologies are not only changing our genome sequencing approaches, but also developing many exciting fields such as metagenomics, meta-transcriptomics and single-cell genomics. Metagenomics is defined as the direct genetic analysis of genomes contained with an environmental sample and produces a taxonomical profile of the sample. An advent of metagenomic analysis coined the term "microbiota" and "microbiome" for microbial community study. Microbiota is defined as the microbial taxa that are associated with an environment and are revealed by using molecular techniques i.e. 16S rRNA gene sequencing. Microbiome refers to microbiota and their genes as well as their products in a given milieu [9]. Furthermore, some researchers add the host and environmental factors in the term microbiome. Meta-transcriptomics is the sequencing of the transcriptome (mRNA) that permits us to evaluate the gene expression of the microbial community. Deep sequencing of DNA and RNA from a single cell, single-cell genomics, let us extensively investigate the cellular functions. Currently, almost all microbial ecology studies employ high-throughput DNA sequencing to explore microbial communities and combine with other platforms such as proteomics or metabolomics to investigate a functional part of microbial communities.

At presence, researchers have a large choice in formulating methodological strategies: depending on the access to the technology, budget, and objectives of research. Each culture-independent methodology has its own limitations and biases. Investigators must take additional measures for example one may use more than one molecular technique or a culture-dependent approach in parallel to provide additional validation of results and reduce the possibility of false findings due to methodological errors and biases. Although the culture-independent techniques have the ability to detect more microbes than culture technique, the culture-dependent methods so far remain a better means of obtaining individual isolates contributing and obtaining isolates for further assays.

The sinonasal microbiota in chronic rhinosinusitis

As the gate into our body, the respiratory tract itself harbors a heterogeneous microbiome that decreases in biomass from upper to lower tract [10]. The sinonasal cavity, the anatomical region responsible for initially filtering the inspired air, is heavily colonized by microbes. The development of culture-independent molecular techniques allows the detection of more bacteria and reveals greater biodiversity than conventional culture [11,12]. Thus, the etiology of CRS may be polymicrobial and the role of anaerobe bacteria may be more prominent than presumed. The culture-independent molecular techniques are enhancing our ability to understand the bacterial community in pathologic condition. However, it is likely that the bacteria detected by culture-dependent techniques still are of clinical relevance to identify specific pathogens in order to direct antibiotic therapies and eradicate bacteria[13].

An important step for a meaningful evaluation of the sinonasal microbiota is to obtain a suitable biological sample. Specimen collection is one of the most important steps in the analysis of areas that are difficult to access, such as the sinuses. It must not be biased by interference from the nares. Specimen can be tissue, nasal secretions, or material sampled by a swab. Researchers have investigated the suitable specimens for microbiota studies in CRS. They found that there is no significant difference between simple swabs and mucosa tissue samples as sampling techniques [14,15]. The use of an endoscope for the sampling is

recommended, although simple swabs are often used in both healthy subjects and diseased patients. Samples can be collected from various anatomical locations in the nose such as the inferior turbinate, the middle-meatus, the ethmoidal sinuses, the sphenoid, and the anterior nasal cavity. A study of variation in the microbial community of the intra-nasal site revealed a variation in microbial community according to site epithelium type in the sinonasal environment [16]. There is a difference in community composition between squamous epithelium (i.e., anterior nares) and ciliated pseudostratified columnar epithelium (i.e., middle meatus, and sphenoethmoidal recess). The middle meatus, delineated/bordered by the middle turbinate and lateral nasal wall, receives drainage from the maxillary, anterior ethmoid, and frontal sinuses. The sphenoethmoidal recess is located in the posterior-most aspect of the nasal cavity, between the nasal septum and superior turbinate that receives drainage from the posterior ethmoid and sphenoid sinuses.Microbial composition at middle meatus and sphenoethmoidal recess is nearly identical (Fig 1)[16]. This indicates that collecting middle meatus swabs is an appropriate sampling technique for DNA-based bacterial assays of paranasal sinuses. This statement is also supported by cultivation-based studies. The microbiology of the middle meatus correlates well with a pathogenic organism of CRS disease [17], whereas swabs of the nares would not be appropriate as a replacement of middle meatus swabs in investigations of CRS pathogens. Researchers can use appropriate protective devices such as a sterilized Killian nasal speculum with long leaves to avoid contamination by the nasal vestibule microbes.

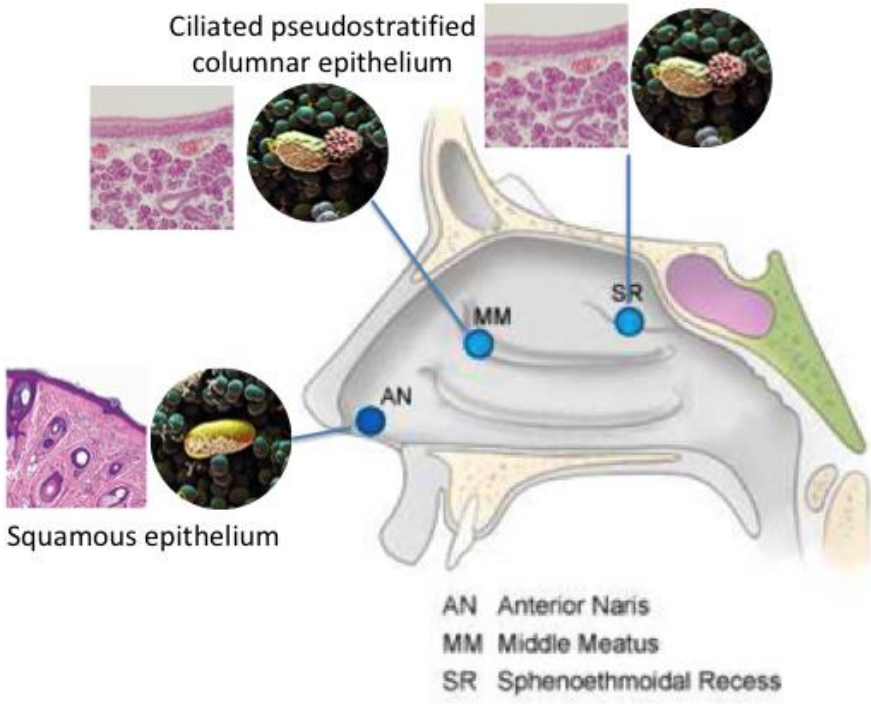

Figure 1. The scheme indicates epithelium type, bacterial communities and locations of the anterior naris, middle meatus, and spenoethmoidal recess in the human nasal cavity.

(Adapted from Yan M, Pamp SJ, Fukuyama J, et al. Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and *S. aureus* carriage. Cell Host & Microbe. 2013)

The traditional view that the paranasal sinuses were an aseptic condition in a healthy person has been largely abandoned. The presence of viable bacteria in healthy sinuses is now well-documented [18]. Early studies using culture-based techniques have now been replaced in the research setting with detection and identification of microbes using nucleic acid-based methods, which appear to be less biased and more sensitive than traditional culture. Before the era of culture-independent methods, conventional cultures have implicated *Staphylococcus aureus,* and *Pseudomonas aeruginosa* as principal pathogens in CRS disease [19]. At the start of the molecular era, researchers employed culture-independent methods to characterize the bacterial communities in CRS patient. Molecular techniques were more sensitive than culture techniques, however, they did not identify the novel organisms implicated in the etiology of CRS context [11,12]. There is no current consensus on the most common bacteria present in the healthy or diseased state. There is no clear 'causative' or 'protective' single organism. However, some commonalities have been identified in multiple studies. The sinus microbiota of CRS patients exhibit significantly reduced bacterial diversity compared to those of healthy control subjects. *Propionibacterium acnes, S. epidermidis, S. aureus* and *Corynebacterium spp.* have been frequently identified as prevalent and abundant species in sinonasal microbiota. Furthermore, a number of particular species commonly viewed as being pathogenic, including anaerobic microbes, *P. aeruginosa* and *Haemophilus spp.*, have been observed in both healthy and CRS subjects. Table 2 provides the studies that using high throughput sequencing of the bacterial 16S rRNA gene for the identification of microbiota in CRS context.

Summary and perspective of nasal microbiota studies

Prior airway microbiota studies revealed several critical factors. It is likely on an account of the substantial technical variation in the methodological approaches employed by different studies. Differences in a patient population, sampling sites and techniques, target gene regions, sequencing platforms, bioinformatics pipelines, and taxonomic assignment databases may have an impact on microbial evaluation; however, a few patterns do emerge. Actinobacteria (i.e., *Corynebacterium spp.* and *Propionibacterium spp.*) have been identified as prevalent and abundant microbes in healthy control subjects. CRS patients often have lower bacterial diversity than that in healthy control subjects. *S. aureus* is detected to be significantly more prevalent and abundant in the CRS groups versus control groups. Although the composition of the microbiota in healthy control subjects and CRS patients is increasingly well-described, the role of the microbiota in the pathogenesis of CRS disease remains unclear. A study with well-defined patients, using phenotypes and potentially endotypes of CRS disease can possibly identify signature microbiota, which might be related to specific pathological or immunological characteristics of the inflammation. Research on how microbiota impact on the immune response of nasal and sinus mucosa may shed a new light on the pathophysiology of CRS disease and consequently may result in new strategies for its treatment.

Table 2. Literature summary of the microbiota studies using high throughput sequencing technique in chronic rhinosinusitis.
Legend: all studies were carried out with Roche FLX, except for Cope et al. [20], on Illumina MiSeq

Study	Year	Design	Sample type	Reference data base	Main genera identified:
Feazel et al [21]	2012	2 CRSwNP 13 CRSsNP 5 controls	Swab to areas of mucopurulence when present or middle meatus when no gross disease present	Silva	*Corynebacterium* *Propionibacterium* *Staphylococcus*
Aurora et al [22]	2013	20 CRSwNP 10 CRSsNP 12 controls	Nasal lavage fluid	Silva	*Corynebacterium* *Curtobacterium*
Liu et al [23]	2013	4 CRSwNP 2 CRSsNP	Maxillary sinus swab	Ribosomal Database Project	*Haemophilus* *Prevotella* *Pseudomonas*
Choi et al [24]	2014	5 CRSwNP 3 CRSsNP 3 controls	Nasal lavage fluid	ExTaxon	*Prevotella* *Staphylococcus*
Kim et al [14]	2015	5 CRSwNP 4 CRSsNP	Paired middle meatus swab and mucosal biopsy	Silva	*Corynebacterium* *Peptoniphilus* *Prevotella* *Staphylococcus*
Hauser et al [25]	2015	21 CRSwNP 33 CRSsNP	Ethmoid sinus swab	Silva	*Anaerococcus* *Corynebacterium* *Propionibacterium* *Ralstonia* *Staphylococcus* *Stentrophomonas* *Streptococcus*

Study	Year	Design	Sample type	Reference data base	Main genera identified:
Biswas et al [26]	2015	2 CRSwNP 6 CRSsNP 1 CRS+CF 6 controls	Swab from left and right sides of nostril, anterior nares, inferior turbinate, and middle meatus	Silva	*Corynebacterium* *Staphylococcus*
Bassiouni et al [27]	2015	4 CRSwNP 2 CRSsNP	Paired ethmoid sinus swabs and mucosal tissue biopsies	Greengenes	*Anaerococcus* *Corynebacterium* *Propionibacterium* *Pseudonocardis* *Staphylococcus*
Ramakrishnan et al [28]	2015	22 CRSwNP 48 CRSsNP 31 controls	Middle meatus swabs	Silva	*Clostridium* *Corynebacterium* *Propionibacterium* *Ralstonia* *Staphylococcus*
Liu et al [29]	2015	14 CRSwNP 28 controls	Nasal cavity and maxillary sinus mucosa swab	Ribosomal Database Project	*Corynebacterium* *Finegoldia* *Serratia*
Ramakrishnan et al [30]	2015	21 CRSwNP 35 CRSsNP 26 controls	Ethmoid swab, a neighboring sinus, or both when purulence was present	Silva	*Corynebacterium* *Propionibacterium* *Staphylococcus*
Cope et al [20]	2017	27 CRSwNP 23 CRSsNP 9 CRS+CF 10 controls	Sinus brushings	MicroNorm	*Corynebacterium* *Pseudomonas* *Staphylococcus* *Streptococcus*

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Chapter 5.
Aims of the study

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The etiology of chronic rhinosinusitis (CRS) remains only partially understood. CRS is a heterogeneous condition characterized broadly by persistent inflammation of the sinonasal mucosa. The causes of inflammation in CRS are diverse and multifactorial, relating to overlapping host and environmental triggers. Microorganisms (i.e., bacteria, and fungi) have long been considered as an inflammatory initiator, or at least contribute to its persistence. Modern culture-independent techniques have vastly improved our understanding of the complex microbial communities associated with the human body. It has been suggested that CRS occurs as the result of an inappropriate or exaggerated response to external environmental triggers. Therefore, local environmental factors e.g. resident microbes may contribute to the pathogenesis of CRS.

Aims of this thesis

The aim of the work described in this thesis was to determine the possible interaction between microbiota and the mucosal immune reaction, and thus on the development of inflammation in chronic rhinosinusitis. The study aims:

1) To investigate the association between mucosal microorganisms and inflammatory patterns, using peptide nucleic acid fluorescence in situ hybridization (PNA-FISH).

2) To compare the composition and diversity of the nasal microbiota of healthy controls to the composition and diversity of the nasal microbiota of patients with CRSwNP, employing high throughput sequencing of the bacterial 16S rRNA gene.

After identifying the microorganisms, we attempted to investigate the role they play in human health and disease.

Chapter 6.
Association of Mucosal Organisms with Patterns of Inflammation in Chronic Rhinosinusitis

Chapter 6. Association of Mucosal Organisms with Patterns of Inflammation in Chronic Rhinosinusitis

Based on: Chalermwatanachai T, Zhang N, Holtappels G, Bachert C.
Association of mucosal organisms with patterns of inflammation in chronic rhinosinusitis. PloS One. 2015;10(8):e0136068.

Introduction

Chronic rhinosinusitis (CRS) can be classified into two clinical subgroups (phenotypes) based on the absence and presence of nasal polyps (CRSsNP and CRSwNP) [1]. These two subgroups may be further divided into several disease endotypes based on the cytokine composition predominant within the tissue [2]. The phenotype is useful in treatment planning while the endotype provides insight in pathophysiological mechanisms of CRS. Therefore, investigations of the pathogenesis and the factors amplifying mucosal inflammation are of crucial importance for the development of new diagnostic and therapeutic tools.

In our previous findings, using the peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH), we reported a higher quantity of *Staphylococcus aureus* in nasal polyps (NPs) tissues from patients with aspirin exacerbated respiratory disease [3]. We also found an association between gram-positive bacterial colonization and interleukin (IL)-5-positive NPs, whereas gram-negative bacteria were associated with IL-5-negative NPs [4]. Thus, specific inflammatory endotypes are likely to be associated with the intramucosal presence of specific bacteria but it remains unclear which organisms are related to the different endotypes.

Microorganisms have a possible influential role, at least in selected subsets or endotypes of CRS; mucosal bacteria might have a specifically direct and strong impact on the mucosal immune system. To validate this hypothesis, we attempted to illustrate the association of specific microorganisms and specific inflammatory patterns. The main emphasis of the current study was on intra-mucosal microorganisms such as *S. aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, and fungi, all detectable by the PNA-FISH.

Materials and Methods

Patient groups and samples

Sinonasal tissue samples (n=115) with defined disease and pathology were selected from a sinonasal tissue collection at the Department of Otorhinolaryngology, Ghent University Hospital. These sinonasal tissues were obtained from patients in the academic center who probably had prolonged medical treatment. The study for collecting human sinonasal tissue samples was approved by the ethics committee of the University of Ghent, Belgium and appointed the number B67020111209. All patients gave written informed consents before being operated on. These sinonasal tissue samples were obtained from patients during endoscopic sinus surgery. The inferior turbinates from patients undergoing septoplasty without sinus disease were used as control. The diagnosis of CRS in each patient was in compliance with the EPOS guidelines. Diagnosis of cystic fibrosis (CF) and asthma was subsequently confirmed by a pediatrician/pulmonologist. The atopic status was evaluated using the skin prick tests to common inhalant allergens. Cytokine profiles (Box 1) were performed on all disease tissue samples. Sinonasal tissue samples were classified into five groups according to their cytokine composition: sinonasal tissues from CRSsNP patients with tumor necrosis factor alpha (TNFα) mucosal concentrations < 20 pg/ml or > 20 pg/ml, sinonasal tissues from CRSwNP patients with IL-5 and SE-IgE expression, sinonasal tissues from CRSwNP patients without IL-5 and SE-IgE expression, and sinonasal tissues from patients with CF.

Box 1. Cytokine profiles and their function

Cytokine profile	Interpretation of increased concentrations
TNFα	Proinflammatory action
IFNγ	Th1 activity
IL-5	Th2 activity
SE-IgE	Marker for superantigen effect on local mucosa
IgE	Adaptive immunity marker
ECP	Eosinophilic activity marker
IL-22	Th22 activity
IL-17A	Th17 activity

Measurement of Cytokines in Tissue

Snap-frozen tissue specimens were weighed and suspended in a ratio 0.1 g of tissue per 1 mL of 0.9% NaCl solution with a complete protease inhibitor cocktail (Roche; Mannheim, Germany). To prepare soluble protein fractions, the frozen tissues were pulverized using mechanical Tissue Lyser LT (Qiagen Hilden, Germany) at 50 oscillations per second for two minutes in prechilled Eppendorf tubes. Homogenized tissues were centrifuged at 1,800x g for five minutes at 4°C, and the supernatants were collected. Total IgE, eosinophil cationic protein (ECP) and specific IgE to staphylococcal enterotoxins (SE-IgE) were measured using the UniCAP system (Phadia; Uppsala, Sweden). Assay of the TNFα, interleukin (IL)-5, and IL-17 were performed using Luminex (R&D system; Minneapolis, MN, USA). Interferon gamma (IFNγ) was determined using ELISA (R&D system; Minneapolis, MN, USA). IL-22 was assayed using Duoset-Elisa from R&D system.

Identification of Microorganisms by Peptide Nucleic Acid Fluorescence in situ Hybridization

Sinonasal tissues were prepared using frozen section procedure and cut to 5 μm, fixed with 70% ethanol for ten minutes and air-dried. Subsequently, the dried sections were hybridized at 55 °C for 90 minutes in a humidified chamber with 100–500 nM of fluorescein-labeled PNA probe and hybridization buffer. All the PNA probes and buffer solutions were taken from the PNA-FISH kit from AdvanDx; Woburn, MA, USA. We employed Uniprobe to hybridizes with a wide range of

gram positive and gram negative bacteria. For *S. aureus*, *P. aeruginosa* and *E. coli* we used appropriate probes. A panFungal PNA probe was used to detect a wide range of fungi. After hybridization, the coverslips were removed by submerging each slide in the stringent wash buffer and washed for 30 minutes in a shaking water bath at 55°C. Each slide was subsequently mounted in Vectashield (Vector; Burlingame, CA, USA) containing 4,6-diamidino-2-phenylindole dihydrochloride (Roche Molecular Biochemicals; Brussels, Belgium) to counterstain the nuclei. A negative control (without PNA probe) was run in parallel to each sample. Microscopic examination was conducted with a Zeiss Axioplan epifluorescence microscope (Carl Zeiss; Gottingen, Germany) equipped with a CCD camera (IMACCCD S30; SONY, Germany) using a fluorescein isothiocyanate specific filter. Images were captured using the Isis imaging and software system (MetaSystems; Sandhausen, Germany). The samples were assessed using a score of bacterial appearance in the mucosa from 0 to 3: 0, negative; 1, extraepithelial presence; 2, intraepithelial presence and 3, subepithelial presence (Fig 1). Ten high-power fields were counted and added resulting in a total mucosal score between 0 and 30. Furthermore, we interpreted mucosal invasion as; negative (all 10 fields scored 0), noninvasive (at least 1 field scored 1), or intramucosal invasive (at least 1 field scored 2 or 3) based on the maximum score. Two independent observers, not privileged to the diagnosis and clinical data, evaluated the slides.

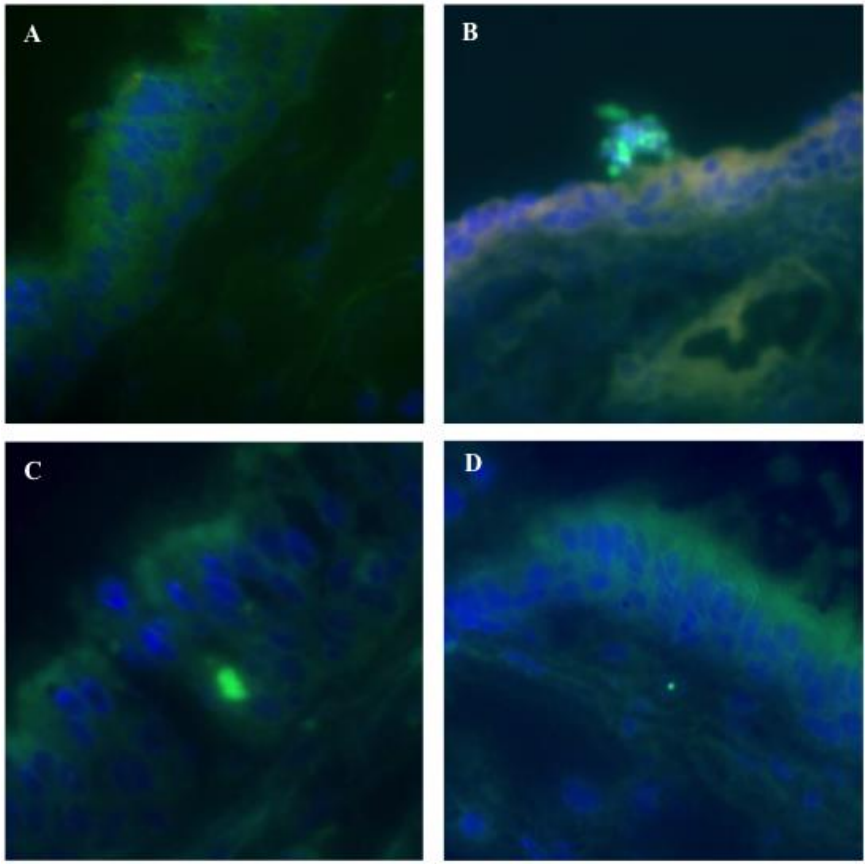


Figure 1. Detection of microorganisms in sinonasal tissue with peptide nucleic acid-fluorescence *in situ* hybridization. The figures show autofluorescence of the sinonasal epithelium; without the postive staining (A), with extraepithelial presence (B), with intraepithelial presence (C), and with subepithelial presence (D). (magnification 100x).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 6.00 for Mac OS X (GraphPad Software; La Jolla CA, USA, www.graphpad.com). The categorical data (sex, positive atopy, asthma, and probe) were expressed as frequencies or percentages and were analyzed using the Chi-square test. The interval data (age, level of cytokines, and total mucosal score) were tested for distribution using the Shapiro-Wilk normality test revealing that the data were distributed non-normally. The Kruskal-Wallis test was used to assess the significance of intergroup correlation, and corrections of significance for between-group comparisons were calculated using Dunn's test. The Spearman's rank correlation was employed to assess the statistical correlation between cytokine and mucosal score. The statistical significance level was determined as $p < 0.05$.

Results

Characteristics in the Nasal Tissue Samples

Table 1 illustrates the number of samples and clinical characteristics of the patients. More than half (62.5%) of the CRSwNP patients with IL-5 and SE-IgE positive had co-morbid asthma. CF patients were the youngest as expected. No significant differences were found with regard to sex and atopy in intergroup comparisons of patients.

Table 1. Sample characteristics

	Control	CRSsNP TNFα<20	CRSsNP TNFα>20	CRSwNP IL5&SE-IgE Neg	CRSwNP IL5&SE-IgE Pos	Cystic Fibrosis	Statistic significance (*P*)
No. of samples	20	20	20	20	20	15	
Mean age(yr)	32.75	44.45	39.35	41.05	51.2	13.25	< 0.0001
Female/male	5/15	11/9	11/9	10/10	8/12	6/9	NS (0.3691)
Atopy positive	5.88%	20%	31.25%	29.41%	52.94%	n/a	NS (0.6776)
Asthma	0 %	20%	20%	35.29%	62.50%	n/a	0.0330

Cytokine Patterns in the Sinonasal Tissue Samples

Typical patterns of cytokines of sinonasal tissues from CRSsNP patients are T helper 1(Th1)-driven, whereas sinonasal tissues with eosinophilia from CRSwNP patients are T helper 2(Th2)-biased. Non-eosinophilic sinonasal tissues from CRSwNP patients express increased amounts of IFNγ and/or IL-17 or none of those. Sinonasal tissues from CF patients demonstrate high concentrations of IL-17 and IL-8 [5]. In the current study, concentrations of ECP and IgE were significantly higher in sinonasal tissues from CRSwNP patients with IL-5 expression than those all other groups. Additionally, an increase in SE-IgE concentration was observed. The number of SE -IgE positive subjects was significantly higher in this group than those in the controls and the other groups. TNFα was detected in sinonasal tissues from CRSsNP patients and CRSwNP patients with IL-5 positive tissues. IFNγ was noted in the sinonasal tissue homogenates with a concentration of TNFα >20 pg/ml from CRSsNP patients, IL-5 negative sinonasal tissues from CRSwNP patients, and sinonasal tissues from CF patients. The expression of IL-22 showed a significant increase in the TNFα >20 pg/ml sinonasal tissue homogenates from CRSsNP patients compared with those of all other groups. A remarkably decreased level of

IL-22 was observed in sinonasal tissues from CF patients compared with those in the control and sinonasal tissues from CRSsNP patients. Although IL-17 expression was higher in sinonasal tissues from CF patients, it did not reach the level of statistical significance (p = 0.0545)(Fig 2).

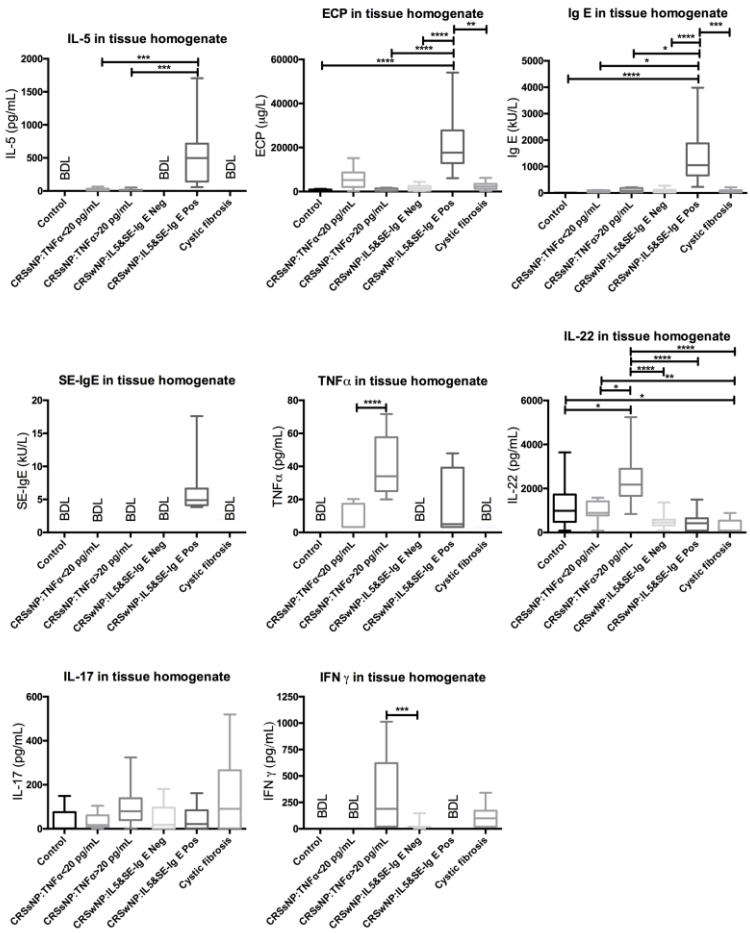


Figure 2. Cytokine expression in sinus tissues. The graphs represent median, upper and lower quartiles. High IgE, IL-5 and ECP concentrations characterize the inflammation in CRSwNP:IL5&SE-IgE positive. In CRSsNP: TNFα >20 pg/mL, the expression of IFNγ and IL-22 are elevated.

Statistical analysis was performed using Kruskal-Wallis and Dunn's multiple comparisons test. *: p value ≤ 0.05. **: p value between 0.001 and 0.01, ***: p values between 0.0001 and 0.001, ****: p value < 0.0001. BDL stands for Below Detection Limit.

PNA-FISH Probe Results

The detection rate of S. aureus was significantly higher in sinonasal tissues from CRSwNP patients with IL-5 and SE-IgE (75%) and CF patients (60%) than those from the control patients (15%, $p = 0.0001$ and $p = 0.0055$ respectively). The presence of *P. aeruginosa* in samples of sinonasal tissue from CRSsNP patients with TNFα concentrations > 20 pg/ml group (70%) was significantly higher than in the control group (35%, $p = 0.0267$). The PNA-FISH probe for *E. coli* and panfungus revealed no statistically significant difference among all groups. Furthermore, the Uniprobe discovering all bacteria did not show any difference in numbers, indicating that all samples had about the same total bacterial load. Representative PNA probe findings are shown in Figure 3.

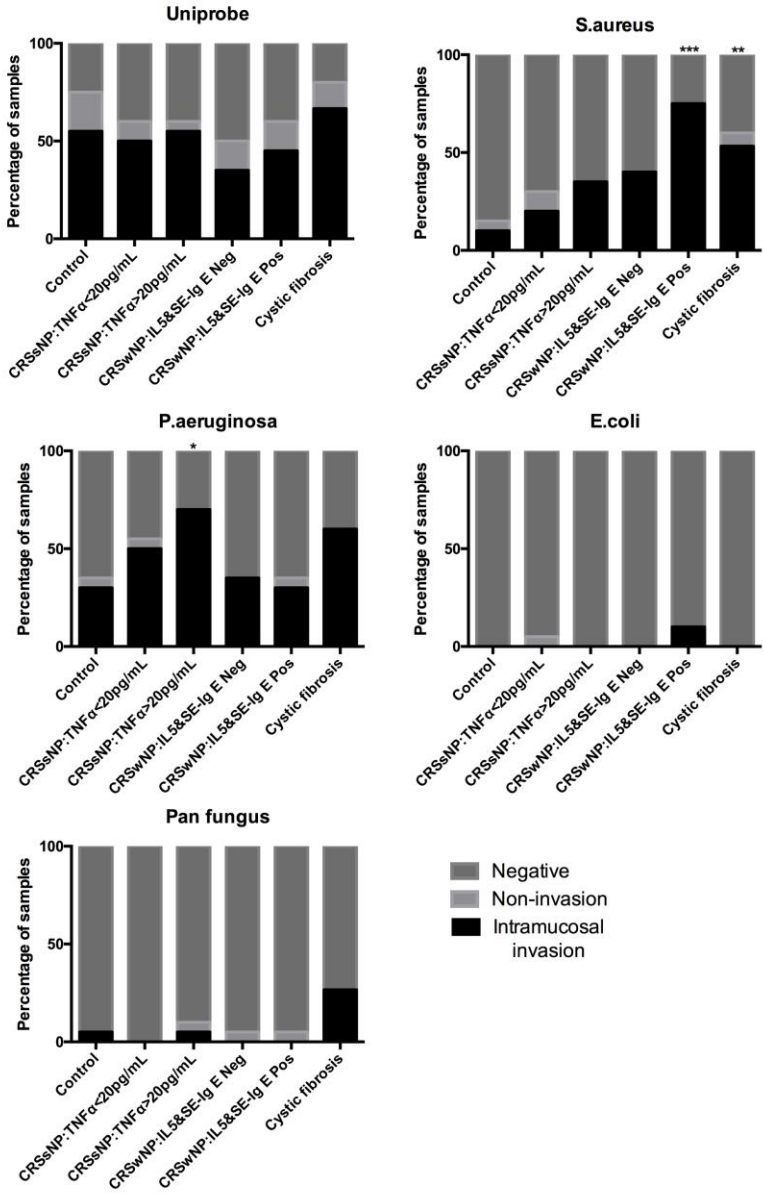


Figure 3. PNA-FISH. Percentages of samples of bacterial presence for each of the probes in the different patient groups. Differences from the control group were calculated using the Chi-square test. *: p value ≤ 0.05. **: p value between 0.001 and 0.01, ***: p values between 0.0001 and 0.001.

The total mucosal scores (median [interquartile range]) for *S. aureus* in the CRSwNP:IL-5 and SE-IgE positive group (3 [0-13]) and CF group (3 [0-19]) were significantly different from that in the control group (0[0-7]). Asthma had no significant impact on the scores in the CRSwNP:IL-5 and SE-IgE positive group. Invasion of the mucosa by *P. aeruginosa* was significantly more frequent in the CRSsNP:TNFα > 20 pg/ml group (median 8 [0-15]) than that in control group (median 0 [0-6]) (Fig 4).

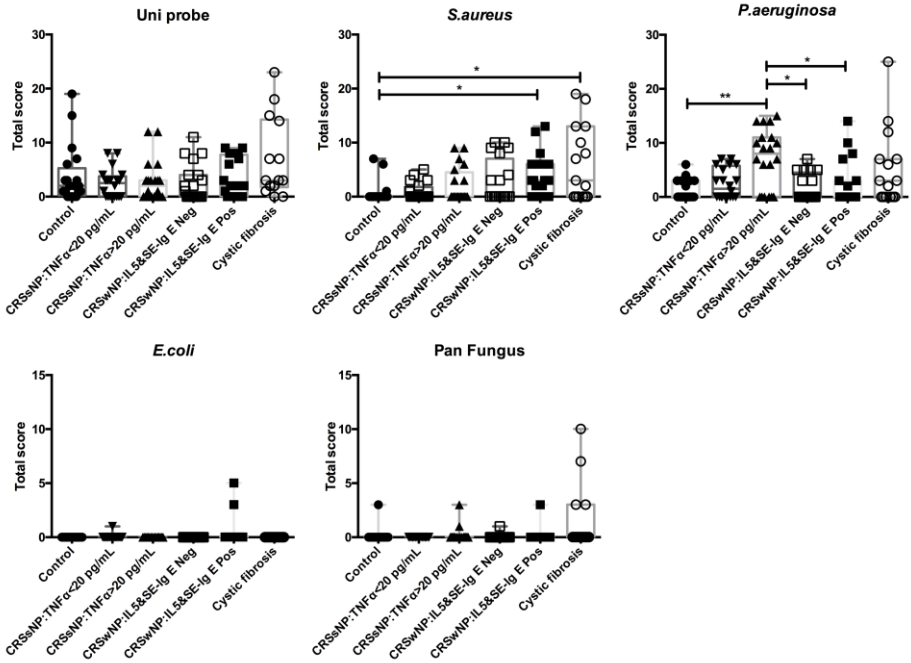


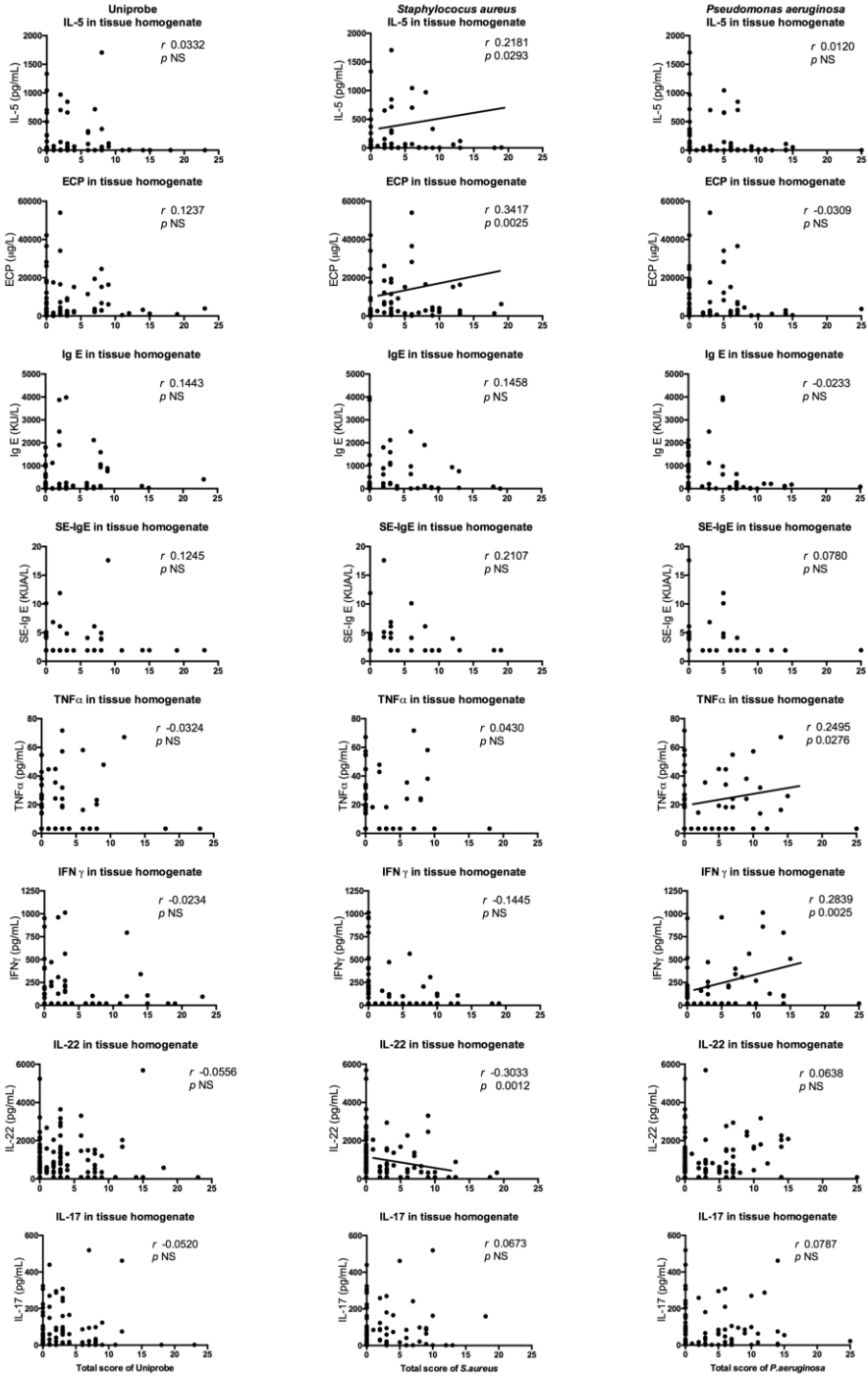
Figure 4. Total mucosal scores for the presence of bacteria in sample tissues, detected by PNA-FISH Each point represents the score from the individual sample. The data for each group are expressed as median and interquartile range. The significance between groups was tested using Kruskal-Wallis test. Dunn's test was employed to correct the significance for between-group comparisons. *: p value ≤ 0.05. **: p value between 0.001 and 0.01.

Correlations between Cytokine Levels and Total Mucosal Score

Correlations of cytokines and scores of bacterial appearance in the mucosa reveal that IL-5 and ECP were positively correlated with the *S. aureus* score (r =0.2181, p = 0.0293; and r = 0.3417, p = 0.0025, respectively). In contrast, IL-22 was negatively correlated with the *S. aureus* score (r = -0.3033, p = 0.0012). The *P. aeruginosa* score positively correlated with TNFα (r = 0.2495, p = 0.0276) and IFNγ (r = 0.2839, p = 0.0025). However, no cytokine detectable in tissue correlated with the Uniprobe score (Fig. 5).

Figure 5. (adjacent page) Correlation between cytokines and total mucosal scores for all bacteria (UniProbe), and for *S. aureus* and *P. aeruginosa*, on the basis of PNA-FISH.

The correlations were calculated using Spearman's rank test. Correlation coefficient (r) and p value were given for each correlation. NS: Not Significant ($p > 0.05$).


Uniprobe
IL-5 in tissue homogenate
r 0.0332
p NS
IL-5 (pg/mL)
Staphylococus aureus
IL-5 in tissue homogenate
r 0.2181
p 0.0293
IL-5 (pg/mL)
Pseudomonas aeruginosa
IL-5 in tissue homogenate
r 0.0120
p NS
IL-5 (pg/mL)
ECP in tissue homogenate
r 0.1237
p NS
ECP (µg/L)
ECP in tissue homogenate
r 0.3417
p 0.0025
ECP (µg/L)
ECP in tissue homogenate
r -0.0309
p NS
ECP (µg/L)
Ig E in tissue homogenate
r 0.1443
p NS
Ig E (KU/L)
IgE in tissue homogenate
r 0.1458
p NS
Ig E (KU/L)
Ig E in tissue homogenate
r -0.0233
p NS
Ig E (KU/L)
SE-IgE in tissue homogenate
r 0.1245
p NS
SE-Ig E (KUA/L)
SE-IgE in tissue homogenate
r 0.2107
p NS
SE-Ig E (KUA/L)
SE-IgE in tissue homogenate
r 0.0780
p NS
SE-Ig E (KUA/L)
TNFα in tissue homogenate
r -0.0324
p NS
TNFα (pg/mL)
TNFα in tissue homogenate
r 0.0430
p NS
TNFα (pg/mL)
TNFα in tissue homogenate
r 0.2495
p 0.0276
TNFα (pg/mL)
IFN γ in tissue homogenate
r -0.0234
p NS
IFNγ (pg/mL)
IFN γ in tissue homogenate
r -0.1445
p NS
IFNγ (pg/mL)
IFN γ in tissue homogenate
r 0.2839
p 0.0025
IFNγ (pg/mL)
IL-22 in tissue homogenate
r -0.0556
p NS
IL-22 (pg/mL)
IL-22 in tissue homogenate
r -0.3033
p 0.0012
IL-22 (pg/mL)
IL-22 in tissue homogenate
r 0.0638
p NS
IL-22 (pg/mL)
IL-17 in tissue homogenate
r -0.0520
p NS
IL-17 (pg/mL)
Total score of Uniprobe
IL-17 in tissue homogenate
r 0.0673
p NS
IL-17 (pg/mL)
Total score of S.aureus
IL-17 in tissue homogenate
r 0.0787
p NS
IL-17 (pg/mL)
Total score of P.aeruginosa

Discussion

In this study, we employed the PNA-FISH assay to detect intra-cellular bacteria in sinonasal tissue from six groups. As a result, we have identified a relationship between intramucosal microorganisms and inflammatory patterns in several subgroups of CRS patients. We demonstrated that *S. aureus* was present in sinonasal tissues from IL-5 and SE-Ig E positive CRSwNP patients and sinonasal tissues from patients with CF. *P. aeruginosa* was specifically found in the sinonasal tissues of CRSsNP patients with TNFα concentrations above 20 pg/ml.

S. aureus, a gram-positive coccus, expresses a range of virulence factors that challenge the immune system [6]. Our results are consistent with earlier findings that *S. aureus* could drive Th2 type inflammation [2,4,7]. The association of *S. aureus* superantigens and CRSwNP disease was recently confirmed in a meta-analysis [8]. Furthermore, our findings confirm that *S. aureus* colonizes the airways of young patients with CF [9]. However, mucosal *S. aureus* enterotoxin-specific IgEs could be found in adult patients with CRSwNP, those antibodies could not be detected in sinonasal of patients with CF. The reasons for this observation may lie in the ability of *S. aureus* to produce enterotoxins or in the immunological environment. For CRSwNP patient, the presence of IL-5 and SE-Ig E but not *S. aureus* in tissue was associated with comorbid asthma [3,10]. Moreover, the level of IL-5 and SE-Ig E was demonstrated to be significantly increased in recurrent versus nonrecurrent CRSwNP disease [11].

P. aeruginosa is a gram-negative, motile rod bacterium. This study revealed the clinically meaningful presence of *P. aeruginosa* in sinonasal tissues of CRSsNP patients with TNFα concentrations higher than 20 pg/ml, coinciding with elevated levels of IFNγ and IL-22. These findings confirm a Th1 milieu in CRSsNP disease and suggest that *P. aeruginosa* may drive Th1 type inflammation and a specific antibacterial response [12]. *P. aeruginosa* does not only stimulate TNFα production in epithelial cells [13], but also induces dendritic cell maturation to the Th1-polarizing phenotype [14]. Moreover, *P. aeruginosa* activates dendritic cells, Th1 cells, and group 3 innate lymphoid cells (ILC) in the mucosa to produce IL-22 [15,16].

IL-22, a member of the IL-10 family [17] is essential for mucosal immunity. It boosts the innate immunity [16] and induces epithelial cells to produce antimicrobial proteins [18]. In murine models, IL-22 was demonstrated to play a vital role in the fight against gram-negative bacteria in the respiratory system [19,20]. The decreased expression of IL-22 in sinonasal tissues from CF patients may predispose these patients to gram-negative infection in adults.

This study illustrated that intramucosal *P. aeruginosa* colonization relates to TNFα and IFNγ levels in the sinonasal tissue. TNFα has been defined as a key regulatory cytokine of inflammatory responses and plays an important role of normal host defense against microorganisms [21]. Our data suggest that either TNFα or IFNγ may serve as markers for *P. aeruginosa* infections in CRSsNP disease.

Using FISH with the Uniprobe, we found equivalent amounts of bacteria in control and diseased tissues. This is in agreement with recent findings on the sinus microbiome, showing similar results [22]. The number of microbiota per surface area may be similar, the richness and evenness of these microbiota differentiate healthy conditions from diseased conditions. The intramucosal presence of bacteria has been not only demonstrated before in disease, but also in healthy conditions [3,23].

The prevalence of fungi in CRS disease has long been considered. In our series, the pan-fungal PNA-FISH probe only detected a small proportion of fungi without differences between groups [24]. Although studies have reported a predominance of fungi in CRS patients [25,26], the prevalence of fungal DNA in CRS patients was shown to be lower than 10% [27]. It should be taken into account that we may have slightly underestimated the number of fungi present due to the failure of PNA-FISH probes to penetrate the rigid fungal cell walls [28]. This study does not support the hypothesis that fungi are principal offenders in the development of CRS disease.

Future directions for research should involve 16S rRNA gene pyrosequencing and quantitative real-time PCR approaches and study the immune proteomes of the germs and the pathomechanisms involved in the crosstalk with the host. Exploration of the relationships between microbes and their impacts on sinonasal immune responses may lead to a greater understanding of the pathogenesis of CRS, and thus, result in new strategies for its treatment.

Conclusion

For the first time, this study identified a relationship between intramucosal microorganisms and inflammatory patterns in subgroups of patients with CRS. Variations of intramucosal bacteria have been identified between the different CRS endotypes. *S. aureus* is associated with Th2-bias CRSwNP tissue and sinonasal tissues from patients with CF while *P. aeruginosa* is related to high inflammatory CRSsNP disease. We propose TNFα and/or IFNγ as a biomarker for CRSsNP disease. The interaction of microbes with the mucosal immune responses should be further evaluated.

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Chapter 7.
Chronic rhinosinusitis with nasal polyps is characterized by dysbacteriosis of the nasal microbiota

Chapter 7. Chronic rhinosinusitis with nasal polyps is characterized by dysbacteriosis of the nasal microbiota

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Abstract

Chronic rhinosinusitis with nasal polyp (CRSwNP) patients are often characterized by asthma comorbidity and a type-2 inflammation of the sinonasal mucosa. The mucosal microbiota has been suggested to be implicated in the persistence of inflammation, but associations have not been well-defined. To compare the bacterial communities of healthy subjects with those of CRSwNP patients, we collected nasal swabs from 17 healthy subjects, 21 CRSwNP patients without asthma (CRSwNP-A), and 20 CRSwNP patients with co-morbid asthma (CRSwNP+A). We analysed the microbiota using high-throughput sequencing of the bacterial 16S rRNA. Bacterial communities were different between the three groups. *Haemophilus influenzae* was significantly enriched in CRSwNP patients, *Propionibacterium acnes* in the healthy group; *Staphylococcus aureus* was abundant in the CRSwNP-A group, even though present in 57% of the patients. *Escherichia coli* was found in high amounts in CRSwNP+A patients. Nasal tissues of CRSwNP+A patients expressed significantly higher concentrations of IgE, SE-IgE, and IL-5 than those of CRSwNP-A patients. Co-cultivation demonstrated that *P. acnes* growth was inhibited by *H. influenzae, E. coli* and *S. aureus*. The nasal microbiotas of healthy subjects are different from those of CRSwNP-A and CRSwNP+A patients. However, the most abundant species in healthy status could not inhibit those in CRSwNP disease.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is defined as a subgroup of chronic rhinosinusitis (CRS) characterized by the presence of fleshy swellings (nasal polyps) that develop in the lining of the nose and paranasal sinuses[1]. Nasal polyps (NP) are believed to arise in the nasal mucosa because of chronic inflammation. In central Europe, CRSwNP is mostly characterized by a moderate to severe T helper type 2 (Th2)-mediated inflammation with hypereosinophilia and increased IgE concentrations. CRSwNP is difficult to treat and recurrences are frequent, despite medical treatment and surgical interventions. In addition, patients suffering from CRSwNP often have comorbid diseases, such as asthma and aspirin intolerance [2,3].

The definitive mechanisms underlying the pathogenesis of CRS remain poorly elucidated [4]. Microbial involvements have been considered to be one of the mechanisms contributing to the inflammation in case of CRS [5,6], whereas in some reports the characteristics of sinus microbiota in CRS were similar to those of control groups [7-9], other analyses, making use of CRS phenotypes or endotypes, demonstrated different compositions of resident sinus bacterial communities [6,10]. Our group has previously demonstrated that *Staphylococcus aureus* is able to drive Th2 type inflammation and that it is associated with Th2-biased CRSwNP [5,6,11]. We have also reported that the expression of IL-5 and of IgE against *S. aureus* superantigens (SE-IgE) within polyp tissue is associated with comorbid asthma [12,13]. Furthermore, the levels of IL-5 and SE-IgE were significantly increased in recurrent versus non-recurrent CRSwNP [14] and several studies suggest that alterations in the airway microbiota may be associated with inflammatory processes [6,7,10,15-17]. We therefore reasoned that a study with well-defined subgroups of CRS patients could possibly identify different microbiota, which might be related to specific pathological or immunological characteristics of the inflammation.

Here, we hypothesized that heterogeneousness of CRSwNP with regard to asthma comorbidity could be associated with the presence of compositionally distinct sinus microbiota which affect host immune responses. To validate this hypothesis, we collected nasal swabs to obtain a suitable biological sample from the middle meatus, the common location of nasal polyps [18], from CRSwNP patients

without co-morbid asthma and then compared their sinus microbiota to that of patients with CRSwNP with co-morbid asthma and to that of healthy control subjects, using 16S ribosomal RNA-gene (16S rRNA gene) high throughput sequencing. Furthermore, we studied the *in vitro* inhibitory and/or stimulatory effects of isolated strains of different species on each other, specifically to understand to what extent the species that are most frequently present in control subjects may protect the mucosa against germs most frequent in patients with CRSwNP.

Materials and Methods

Study Design and Population

We evaluated nasal microbiota from non-asthmatic CRSwNP patients, CRSwNP patients with co-morbid asthma, and healthy control subjects. Diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps [19]. Diagnosis of asthma was confirmed by a pulmonologist. The atopic status was evaluated by skin prick tests to common inhalant allergens. The control group consisted of healthy volunteers and patients who were free from rhinosinusitis, asthma, and atopy. Patients less than 18 years of age, subjects with cystic fibrosis, known or suspected immunodeficiency or autoimmune disease, and/ or suspected of the use of systemic antibiotics or oral steroids in the last three months before sample collection, were excluded from this study.

Ethics Statement

This study was approved by the ethics committee of the University of Ghent, Belgium and assigned number B670201422215. All participants provided written informed consents prior to their participation in the project. All experiments were performed in accordance with relevant guidelines and regulations.

Sample Collection

Specimens were collected at the Ghent University Hospital, Belgium. Swab specimens for DNA extraction were obtained with eSwab (COPAN, Brescia, Italy).

Swabs were endoscopically guided to the middle meatus region and rotated at least 3 times. In addition, sinonasal tissue samples were obtained from the patients during endoscopic sinus surgery. All samples were immediately transported to the laboratory and snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Measurement of Cytokines in Sinonasal Tissue Samples

Snap-frozen tissue specimens were weighed and suspended in a ratio 0.1 g of tissue per 1 mL of 0.9% NaCl solution with a complete protease inhibitor cocktail (Roche, Mannheim, Germany). To prepare soluble protein fractions, frozen tissues were mechanically pulverized using a Tissue Lyser LT (Qiagen, Hilden, Germany) at 50 oscillations per second for two minutes in prechilled Eppendorf tubes. Homogenized tissues were centrifuged at 1,800x g for ten minutes at 4 °C, and the supernatants were collected. Total IgE, eosinophil cationic protein (ECP) and specific IgE to staphylococcal enterotoxins (SE-IgE) were measured using the UniCAP system (Thermo Fisher Scientific, Phadia, Uppsala, Sweden). Tumor necrosis factor-alpha (TNFα), interleukin (IL)-5 and IL-17 were quantified using the Bio-Plex 200 System (Bio-Rad, Temse, Belgium).

DNA Extraction and Bioinformatic Analysis

DNA was extracted from the whole swab using the FastDNA Spin kit (MP Biomedicals, Solon, Ohio) according to the manufacturer's instructions. Total DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Isogen Life Sciences, IJsselstein, The Netherlands). The quality of the extracted DNA was evaluated on a 1 % agarose gel. Libraries were prepared as previously described [20], using the primers 27F and 338R [20] for the V1-V2 region of the 16S rRNA gene. Libraries were sequenced on a MiSeq (Illumina, Hayward, California). Each sequence was identified using the SILVA database [21]. Definition of operational taxonomic units (OTUs) and data-set quality filter was performed as previously described [20]. After quality filtering, the dataset was then filtered to consider only those OTUs sequences that were present in at least one sample at a relative abundance of > 0.1% or that were present in all samples at a relative abundance of

>0.001%. All samples were randomly re-sampled to equal the smallest read size of 6,682 reads, using the PhyloSeq package [22] from the free software R package for statistical computing and graphics [23]. All the reads were grouped into 942 sequences. All sequences were assigned a taxonomic affiliation (phylotype) based on the naive Bayesian classification (RDP classifier) [24]. A genus name was assigned to a sequence when only 16S rRNA gene fragments of previously described isolates belonging to that genus and of 16S rRNA gene fragments originating from uncultured representatives of that genus showed ≤ 2 mismatches. Species assignments were performed using the Basic Local Alignment Search Tool (BLAST). Assignation of a name demanded at least 99% sequence identity over 95% of sequence length [25].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla, CA: www.graphpad.com). The categorical outcomes (presence/absence of condition) were expressed as frequencies or percentages and were analyzed using the Chi-square test. The interval data (age, level of cytokines) were tested for distribution using the Shapiro-Wilk normality test. Assessment of the significance of intergroup correlation was performed using the parametric test for Gaussian distributed data and a non-parametric method for non-normally distributed data. The ecological profiles were analyzed using the PAST3 program [26]. All Principal Coordinate Analyses (PCoA) were based on a Bray-Curtis similarity index, which operated at the phylotype level. To test for community compositional differences, permutation multivariate analysis of variance (PERMANOVA) was employed. Similarity Percentage (SIMPER) analysis was used to determine the contribution of each species to the observed dissimilarity between samples and to identify the species that are most important in creating the observed pattern. In each group, the 20 bacterial species with the highest relative abundance were selected. This resulted in 22 different bacterial species for further analyses. The difference of relative abundance was calculated using a nonparametric Kruskal-Wallis test and corrections of significance for between-group comparisons were calculated using Dunn's test. The Spearman's rank correlation was employed

to assess the statistical correlation between cytokine and bacterial relative abundance. The statistical significance level was determined as $p < 0.05$.

Bacterial Cultivation and Interaction Assays

An agar diffusion test (Kirby–Bauer method) was employed to determine *in-vitro* interaction between bacterial strains. The bacterial strains were selected based on metagenomic and clinical data. In a separate group of patients (10 healthy control subjects, 10 CRSwNP-A patients, and 10 CRSwNP+A patients) we found that the majority of *S. aureus* strains isolated from CRSwNP patients expressed accessory gene regulator (*agr*) group I and *agr* group II. Reference strains expressing *agr* group I and II were therefore used in this assay. *Staphylococcus aureus* (gfp RN6390 and ATCC 29213), *Escherichia coli* (O157:H7 strain), *Haemophilus influenzae* (ATCC 49144), *Corynebacterium pseudodiphtheriticum* (CIP 103420T), and *Propionibacterium acnes* (ATCC 6919) were selected for the experiment. Chocolate agar plate inoculation and incubation at 37 ºC with 5% CO_2 was determined to be the culture condition that enabled proficient growth of all bacterial strains. Bacterial colonies were harvested, resuspended in 0.9% NaCl to a turbidity that was adjusted to a 0.5 McFarland standard. To test for evidence of cooperative or competitive interaction of bacteria, twelve culture plates were used, of which six were smeared uniformly with 0.5 McFarland suspensions of each of the six test strains to obtain confluent growth (inoculated plate) and the other six were not inoculated (control plates). After left to dry for 5 minutes, 10 µl of 0.5 McFarland standard suspensions of all six test strains were spotted on all culture plates. Agar plates were inspected at day 1, 2, 3, 5 and 7 for bacterial growth on the spots and also around the spots for the inoculated plates. The amount of growth of the spotted strains on the inoculated plates was compared to that on the control plates. Criteria for interpretation were established based on ecological interactions [27,28]. The nine possible interpretations of the interactions, ranging from mutually beneficial through neutral to spiteful interactions, are outlined in Table 1.

Table 1. Criteria for interpretation of bacterial interaction.

Legend: >: more growth of inoculated strain on inoculated plate than control plate, =: no difference in growth; <: less growth

Bottom growth (inoculated plate)	**Top growth (spots on top of inoculated plate)**		
	>	=	<
>	Mutualism	Possible cooperation	Selfishness
=	Commensalism	Neutralism	Amenalism
<	Altruism	Possible competition	Spite

Results

Characteristics of the Patients

Fifty-eight adults [17 healthy control subjects, 21 CRSwNP patients without asthma (CRSwNP-A), and 20 CRSwNP patients with co-morbid asthma (CRSwNP+A)] were enrolled in the study. Table 2 illustrates clinical characteristics of the participants. No significant differences with regard to age or gender were found among the three groups. Atopic status and aspirin intolerance were significantly more prevalent in the CRSwNP+A group, as expected. According to severity of disease based on visual analogue scales for symptoms (VAS), CRSwNP+A patients had remarkably more trouble with nasal congestion and mucus than CRSwNP-A patients (Fig.1).

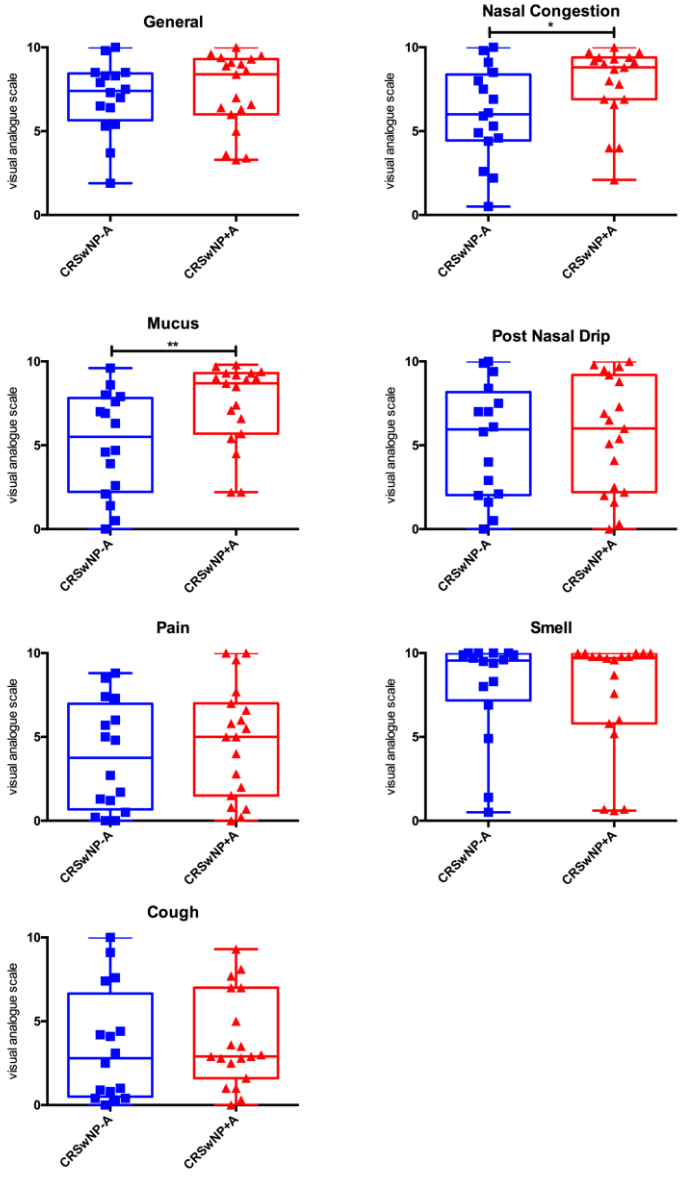

Figure 1. Visual analogue scale. The figure shows the symptom profile and symptom severity of the two patient groups. Nasal congestion and mucus scales were significantly higher in asthmatic patients than non-asthmatic patients. Statistical analysis was performed using Mann-Whitney test.

Legend: *: $p < 0.05$; **: $p < 0.01$.

Table 2. Characteristics of control and patient groups

	Control	CRSwNP-A	CRSwNP+A	Statistical analysis*
Number of subjects	17	21	20	
Mean age (yr)	44.94	47.52	45.75	$p = 0.8688$
Male/Female	9/8	10/11	12/8	$p = 0.7285$
Atopy status positive (%)	0	19	80	$p < 0.0001$
Aspirin intolerance (%)	0	0	33.33	$p = 0.0014$

Legend: *: statistical test used. Kruskal-Wallis test for Age. Chi-square test for gender, Atopy status, and Aspirin intolerance.

Cytokine Patterns in the Sinonasal Tissue Samples

Nasal polyp (NP) disease in European patients is mostly characterized by an infiltration of eosinophils and expression of high concentrations of ECP. In this study, Th2 related mediators and cytokines (IgE, SE-IgE and IL-5) were significantly increased in NP from asthmatic patients compared with those of non-asthmatic subjects, as shown in Fig.2. For example, median total IgE in the CRSwNP-A group was 440.4 kU/L (interquartile ranges IQRs: 101-742.5) compared to 1543 kU/L (IQRs: 640-2321) in the CRSwNP+A group (Table 3). The level of ECP, TNFα, and IL-17 showed no significant differences between groups. Furthermore, the SE-IgE positive group showed notable higher concentrations of IgE (median in the SE-Ig E positive subjects 1307 kU/L vs. 252 kU/L in SE-Ig E negative subjects, $p < 0.0001$), IL-5 (701 pg/mL vs. 77 pg/mL, $p < 0.01$), and ECP (29920 μg/L vs. 14045 μg/L, $p < 0.01$) than in the SE-IgE negative group.

Table 3. Cytokine concentrations of homogenized tissue: CRSwNP-A versus CRSwNP+A

Cytokine (unit)	CRSwNP-A	CRSwNP+A	*p*-value
IL-5 (pg/mL)	63.1 (24.3-150.7)	634.3 (73.6-1639)	0.0018
ECP (µg/L)	15274 (4343-23654)	24486 (10672-32109)	0.0922
Ig E (kU/L)	440.4 (101-742.5)	1543.0 (640-2321)	0.0008
SE-IgE (kU/L)	1.9 (1.9-1.9)	8.0 (5.9-11.4)	<0.001
TNF-α (pg/mL)	25.1 (3.4-48.9)	30.1 (15.8-50.4)	0.7898
IL-17 (pg/mL)	32.0 (1.1-154)	28.4 (1.1-54.7)	0.5628

Values are expressed as median (interquartile ranges); differences between groups are determined using Mann-Whitney test.

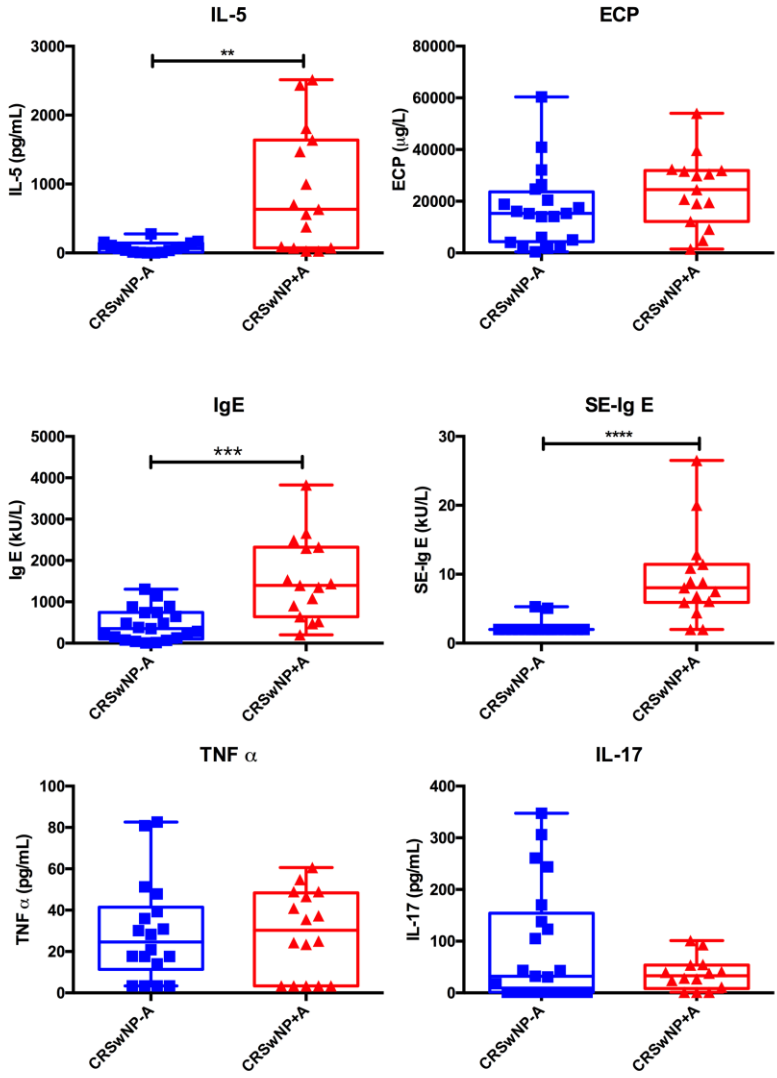

Figure 2. Cytokine expression in nasal polyps tissues. The graphs show median, upper and lower quartiles. High IgE, SE-IgE and IL-5 concentrations characterize the inflammation in NP from asthmatic patients. Statistic results were calculated using the t-test for Gaussian-distributed data and the Mann-Whitney test for non-normal distribution.

Legend: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Microbial Diversity and Relative Abundances of Bacterial Species

Bacterial DNA was detected in all middle meatal swab samples. The number of phylotypes varied from 26 to 207 per sample with an average of 113 phylotypes in the control group, 116 phylotypes in the CRSwNP-A group, and 109 phylotypes in the CRSwNP+A group. Regarding the total number of reads of 16S rRNA gene, there was no statistically significant difference among the three groups, indicating that control subjects and CRSwNP patients had about the same total bacterial load. The sinonasal microbiota of CRSwNP as a group, regardless of co-morbid asthma, however showed significantly decreased bacterial diversity (Shannon H index: a mathematical measure of species diversity in a given community based on the species richness and species abundance) and evenness (Pielou's evenness index: how close in numbers each species in an environment is.), when compared with those of the control group. Especially, the CRSwNP-A group exhibited significantly reduced bacterial evenness and Shannon's diversity when compared with the control group. No significant differences were found with regard to Chao-1 species richness indexes (the number of unique species per sample) in intergroup comparisons (Fig.3).

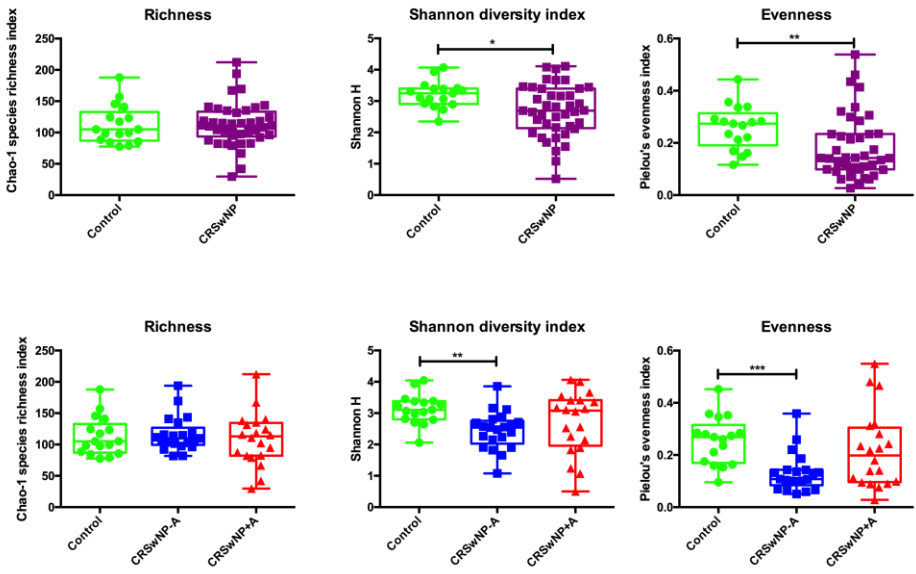

Figure 3 Biodiversity indices. The graphs compare diversity indices (richness, Shannon diversity, and evenness) between healthy control subjects and patients with CRSwNP-A and CRSwNP+A. Statistical analysis was performed using Kruskal-Wallis and Dunn's multiple comparisons test.

Legend: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

The phylum-level structure is depicted on Fig.4. With respect to health and disease status, the genus *Haemophilus* was more abundance in CRSwNP disease than healthy control, while the genus *Bacteroides* was dominant in healthy control subjects when compared with CRSwNP cases. At the species level, *Haemophilus influenzae* was significantly more abundant in CRSwNP patients than in healthy control subjects (Fig. S1a). *Corynebacterium pseudodiphtheriticum* was more prevalent in diseases when compared with controls. *Staphylococcus xylosus* and *Bifidobacterium longum* were more prevalent and abundant in the control group than in the CRSwNP group.

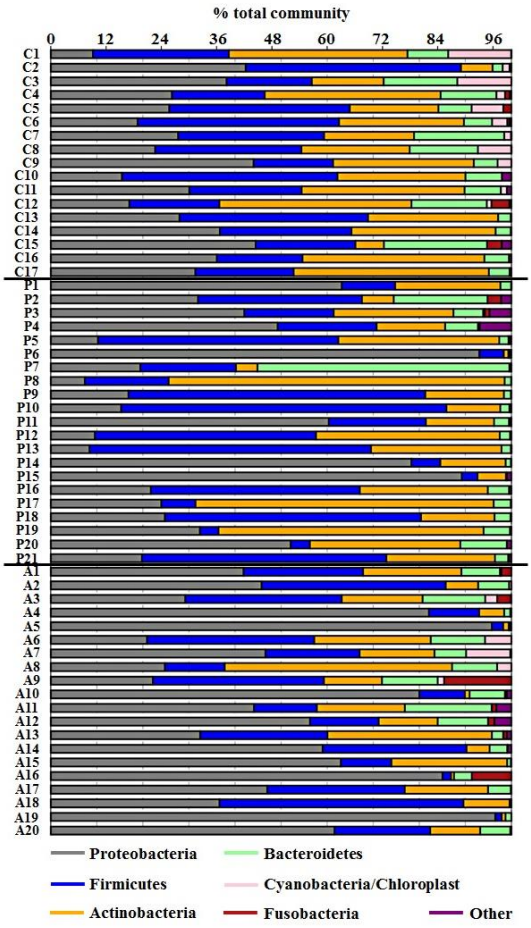

Figure 4. Bacterial community composition in each subject at the phylum level.
Legend: C: healthy control subject, P: CRSwNP-A subject, A: CRSwNP+A subject.

Principal coordinate analysis (PCoA) ordination depicted sinus bacterial beta diversity between samples (Fig.5). One-way PERMANOVA with Bray-Curtis index detected statistically significant differences of bacterial communities in intergroup comparisons (Holm-Bonferroni sequential corrected *p*-values < 0.001). A post-hoc pairwise comparison revealed differences in microbial composition between control and CRSwNP+A groups ($p < 0.01$), control and CRSwNP-A groups ($p < 0.01$), and between CRSwNP patients ($p < 0.03$).

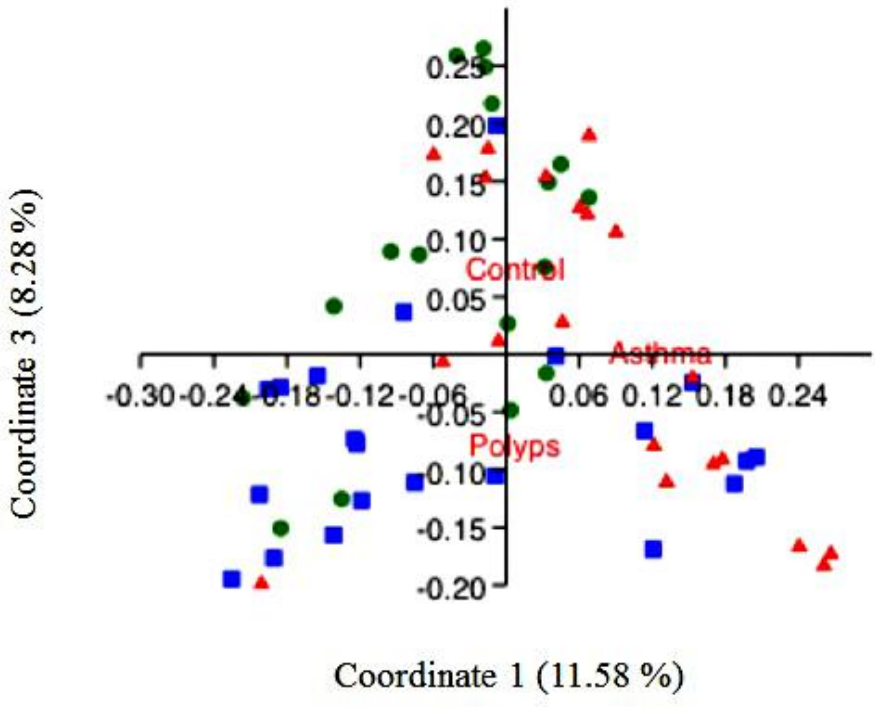

Figure 5. PCoA plot of Bray-Curtis distance between healthy control subjects (green dot), CRSwNP-A patients (blue square) and CRSwNP+A patients (red triangle).

Considering the bacterial composition at the phylum level (Fig.6), Actinobacteria were more abundant in the healthy control subjects than those in patients with CRSwNP+A and the average abundances of Bacteroidetes and Cyanobacteria were notably higher in healthy control subjects than in CRSwNP-A patients. Proteobacteria predominated in CRSwNP+A group compared with CRSwNP-A and control group.

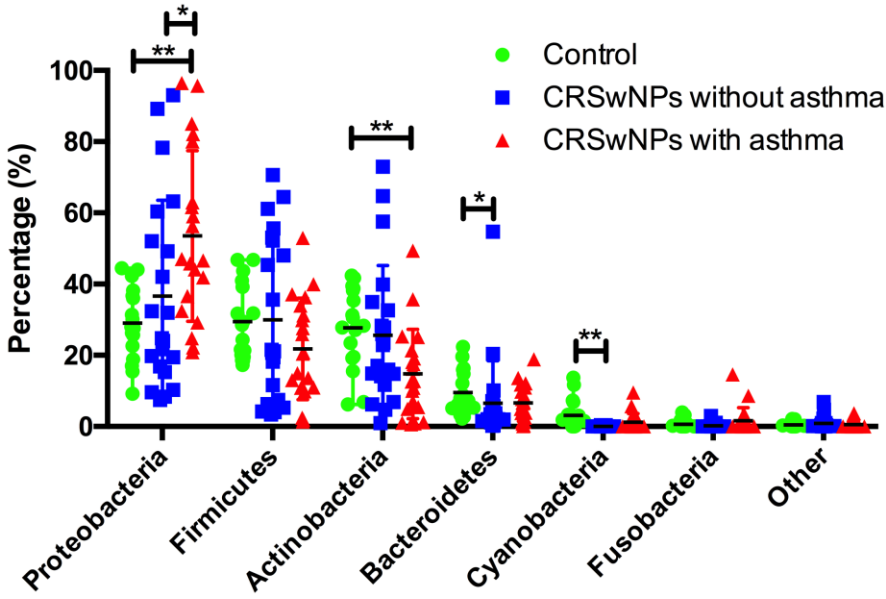

Figure 6. Phylum-level differences between groups. Dot graphs show relative abundance of bacterial phyla from each sample. Kruskal-Wallis and Dunn's multiple comparisons were employed for statistical analysis.

Legend: *: $p < 0.05$; ** $p < 0.01$.

At the genus levels, CRSwNP-A patients carried significantly higher abundance of *Corynebacterium* ($p = 0.04$) (phylum Actinobacteria) and *Geobacter* ($p < 0.01$) (phylum Proteobacteria) than those in CRSwNP+A patients (Fig.7). There was no significant difference between healthy control subjects and CRSwNP-A patients and between healthy control subjects and CRSwNP+A patients.

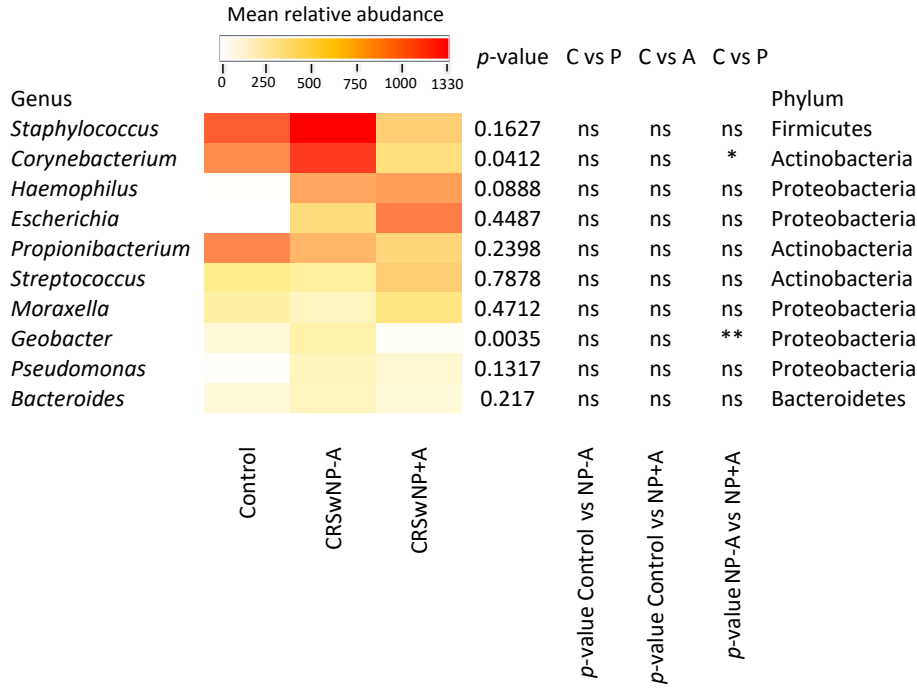

Figure 7. Heatmap analysis of the most abundant bacterial genera. The heatmap shown in white-to-red represents relative abundance that discriminates groups. The difference of abundance was calculated using a nonparametric Kruskal-Wallis test and corrections of significance for between-group comparisons were calculated using Dunn's test. Asterisks denote significant *p*-values.

Legend: *$p < 0.05$, and **$p < 0.01$.

At the species level (Fig.8), CRSwNP+A subjects had a higher prevalent and abundant of *Moraxella catarrhalis* than those in CRSwNP-A subjects, whereas CRSwNP-A subjects had a higher abundance of *Geobacter anodireducens/sulfurrreducens* and *Pelomonas puraquae* than those in CRSwNP+A patients. All aforementioned species are member of the phylum Proteobacteria. CRSwNP-A patients had a significantly lower prevalence of *M. catarrhalis*, *S. aureus* and *Staphylococcus xylosus* than those in the other groups ($p < 0.05$). The mean relative abundance of *S. xylosus* was also notably lower in CRSwNP-A group than those in the other groups. Compared to healthy control group, the prevalence and relative abundance of *Bifidobacterium longum* was lower in CRSwNP-A group.

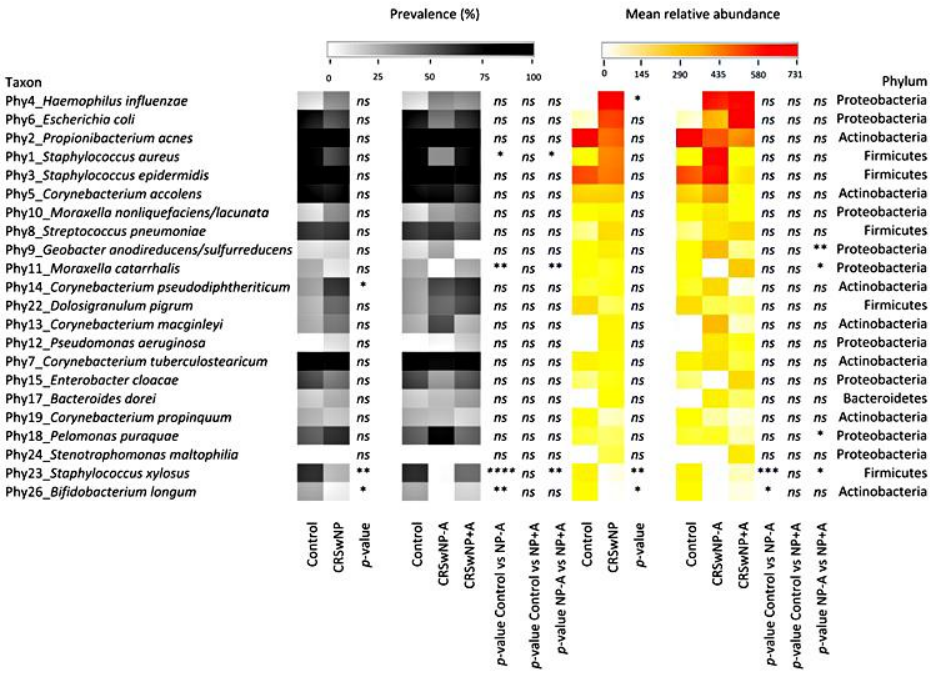

Figure 8. Prevalence and relative abundance of the most abundant bacterial species. The heat maps in white-to-black and white-to-red depict the prevalence and relative abundance, respectively. The order was arranged based on the contribution to the observed dissimilarity.

Legend: *ns*: no statistical significance ($p \geq 0.05$); *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

The prevalence and abundance within bacterial community is summarized in Figure 9. For example, *S. aureus* was prevalent in 94% of healthy control subjects, 57% of CRSwNP-A patients, and 90% of CRSwNP+A patients. The average relative abundance of *S. aureus* was 185 in the healthy group, 654 in the CRSwNP-A group, and 145 in the CRSwNP+A group. In the healthy control group, *Propionibacterium acnes* (phylum Actinobacteria) was the most abundant species. In the CRSwNP-A group had the highest abundance of *S. aureus* (phylum Firmicutes). In the CRSwNP+A group, *Escherichia coli* (phylum Proteobacteria) was found in high amounts.

Taxon	Prevalence (%)					Mean relative abundance				
Phy4_*Haemophilus influenzae*	18	41	18	43	40	0.2	617	0.2	588	647
Phy6_*Escherichia coli*	82	66	82	57	75	35	528	35	345	719
Phy2_*Propionibacterium acnes*	100	100	100	100	100	731	458	731	511	401
Phy1_*Staphylococcus aureus*	94	73	94	57	90	185	405	185	654	145
Phy3_*Staphylococcus epidermidis*	100	98	100	100	95	476	413	476	615	201
Phy5_*Corynebacterium accolens*	88	85	88	90	80	234	236	234	346	120
Phy10_*Moraxella nonliquefaciens/lacunata*	24	46	24	43	50	134	157	134	190	122
Phy8_*Streptococcus pneumoniae*	65	68	65	71	60	69	196	69	184	208
Phy9_*Geobacter anodireducens/sulfurreducens*	18	20	18	33	5	135	163	135	254	69
Phy11_*Moraxella catarrhalis*	29	15	29	0	30	128	112	128	0	230
Phy14_*Corynebacterium pseudodiphtheriticum*	35	66	35	62	70	106	115	106	199	27
Phy22_*Dolosigranulum pigrum*	29	54	35	57	65	192	85	192	91.8	78
Phy13_*Corynebacterium macginleyi*	29	46	29	62	30	11	151	11	253	44
Phy12_*Pseudomonas aeruginosa*	6	20	6	14	25	1	147	1	181	111
Phy7_*Corynebacterium tuberculostearicum*	94	88	94	86	90	150	142	150	186	96
Phy15_*Enterobacter cloacae*	65	51	65	43	60	66	108	66	21	198
Phy17_*Bacteroides dorei*	24	32	24	29	35	2	117	2	156	76
Phy19_*Corynebacterium propinquum*	29	24	29	29	20	127	42	127	56	28
Phy18_*Pelomonas puraquae*	53	66	53	81	50	90	71	90	98	42
Phy24_*Stenotrophomonas maltophilia*	0	2	0	0	5	0	81	0	0	166
Phy23_*Staphylococcus xylosus*	65	24	65	5	45	148	18	148	3	33.5
Phy26_*Bifidobacterium longum*	29	7	29	0	15	133	7	133	0	14
	Control	CRSwNP	Control	CRSwNP-A	CRSwNP+A	Control	CRSwNP	Control	CRSwNP-A	CRSwNP+A

Figure 9 Prevalence and relative abundance within the bacterial community

Correlations between Cytokine Levels and Relative Abundance of Bacteria

Comparison of cytokine levels with relative abundance of bacteria in the nasal polyps tissues revealed that ECP and IL-5 were negatively correlated with the phylum Actinobacteria ($p < 0.05$, r = -0.3652, and -0.3760 respectively). The phylum Bacteroidetes correlated positively with IL-5 ($p < 0.02$, r = 0.3969), but negatively with IL-17 ($p < 0.01$, r = -0.4632).

At the species level, total IgE and IL-5 were negatively correlated with *Geobacter anodireducens/sulfurreducens* and *Pelomonas puraquae*, IL-5 was negatively correlated with *Corynebacterium macginleyi*, and ECP was negatively correlated with *Corynebacterium accolens*, *C. macginleyi* and *Streptococcus pneumoniae*. In contrast, ECP and IL-5 were positively correlated with *E. coli*. *S. aureus* and *P. acnes* were not correlated with any cytokine. IL-17 and TNF-α were not correlated with any of the top 20 bacterial species (Table 4). However, no single correlation reached a correlation coefficient of higher than 0.6 (moderate correlation).

Table 4. Significant correlations between cytokines and relative abundance of top 20 bacterial species in the sinus microbiota of CRSwNP patients

Species	Immune marker: Coefficient (*p*-value)		
	Total IgE	**ECP**	**IL-5**
Bacteroides dorei			0.4472 (0.0062)
Corynebacterium accolens		-0.3880 (0.0213)	
Corynebacterium macginleyi		-0.5658 (0.0004)	-0.4030 (0.0148)
Enterobacter cloacae		0.3393 (0.0461)	0.3377 (0.0439)
Escherichia coli		0.4638 (0.0050)	0.4150 (0.0118)
Geobacter anodireducens/ G. sulfurreducens	-0.4510 (0.0058)		-0.3704 (0.0262)
Pelomonas puraquae	-0.3563 (0.0330)		-0.3526 (0.0349)
Streptococcus pneumoniae		-0.3539 (0.0370)	

Interspecies Interactions of Cultured Bacterial Species

When testing the possible interactions between six strains of five of the most frequently present species, we observed that growth of *C. pseudodiptheriticum* on the inoculated plate was enhanced by the presence of *S. aureus* RN6390 and *H. influenzae*, but retarded by *E. coli* and inhibited by *S. aureus* ATCC 29213. *P. acnes* appeared to be inhibitied by *S. aureus* (both strains), *E. coli* and *H. influenzae*. Furthermore, *H. influenzae* was inhibited by *S. aureus* ATCC 29213 and *E. coli*. Growth of *S. aureus* RN6390 was inhibited by *E. coli*. With respect to growth pattern of the strains used to initially inoculate the plates, *C. pseudodiptheriticum* swiftly flourished in the proximity of the *E. coli* inoculation point, whereas growth of *P. acnes* was inhibited around the inoculation point of *S. aureus* ATCC 29213. Neither growth inhibition nor growth promotion was noticed between *C. pseudodiptheriticum and P. acnes*, and between *S. aureus* ATCC 29213 and *E. coli*. Fig.10 demonstrates the observed bacterial growth patterns. Interspecies interplay on the basis of the available information from our *in-vitro* experiments is depicted in Fig.11 (Fig S2).

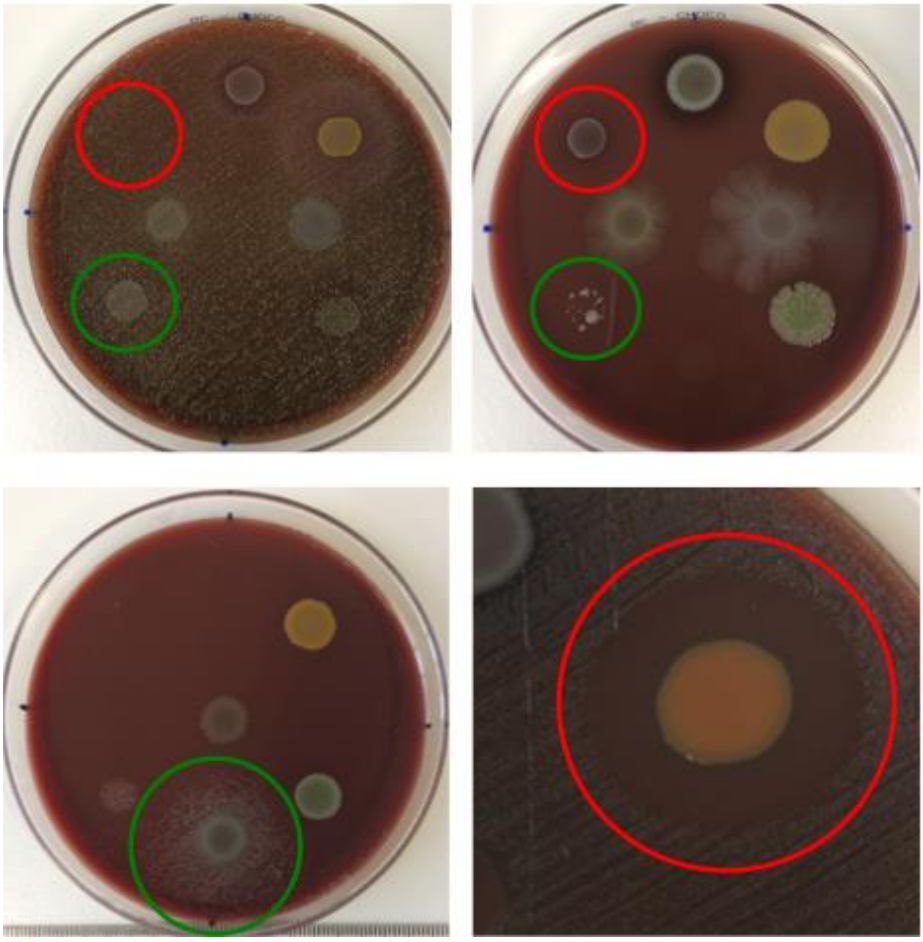

Figure 10. Record of bacterial growth in competitive experiments. Growth promotion and growth inhibition are highlighted by green and red circles, respectively. Growth of an inoculated strain on a smeared strain (upper left photo) compared to control plate (upper right photo): The red circles mark the absence of *P. acnes* growth on *H. influenzae*. The green circles show enhanced growth of *C. pseudodiphtheriticum* on *H. influenzae*. The growth of the smeared strain around the point of inoculation of a second germ is displayed in the lower photos. The green circle (lower left photo) shows enhanced growth of *C. pseudodiphtheriticum* surrounding the *E. coli* spot, whereas the red circle (lower right photo) points to a zone of inhibition of *P. acnes* around the *S. aureus* spot on chocolate agar.

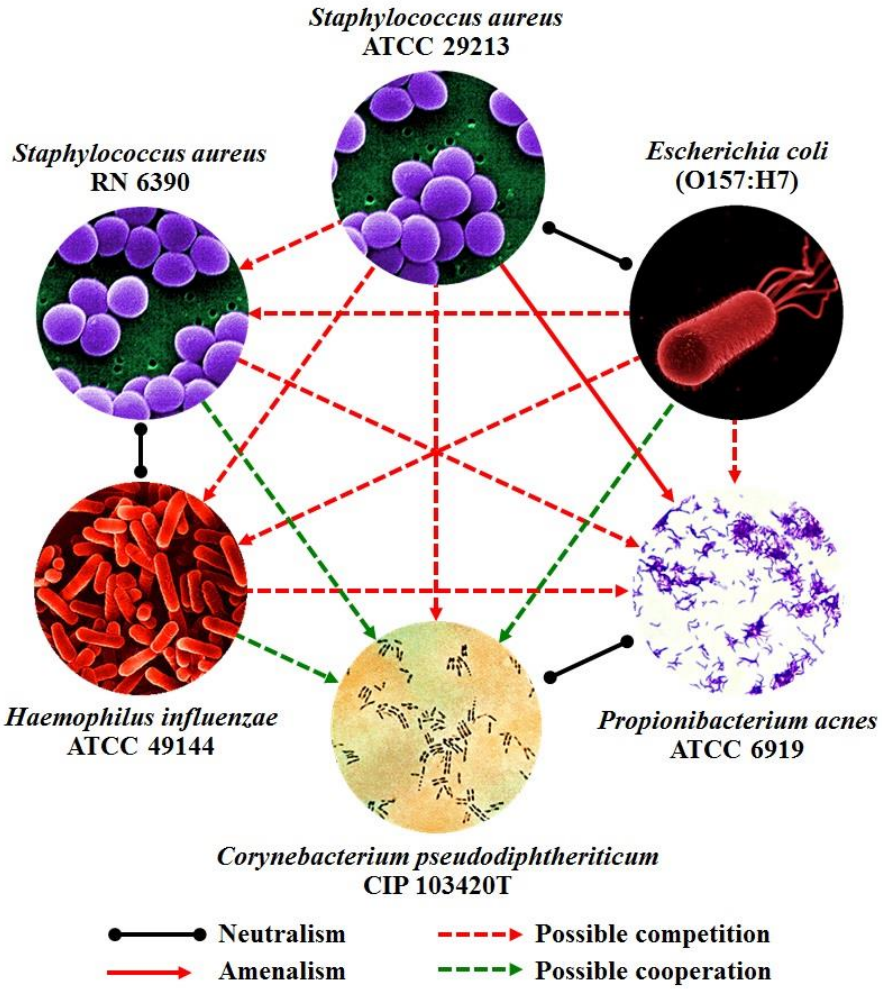

Figure 11. Bacterial in vitro interactions.

Legend: Red lines represent a negative relationship, green lines represent a positive relationship, and black lines represent neutral relationship.

Discussion

The nasal bacterial community has been suggested to play a role in the development and severity of CRSwNP disease [29,30]. The aim of this study was to establish whether and to what extent there might be differences in the nasal microbiota of healthy control subjects, compared to those of non-asthmatic and asthmatic CRSwNP patients. Subgrouping of the patients into endotypes was based on our previous studies [3,6]. Indeed, CRSwNP disease can be further classified on the basis of distinct biomarkers (IgE, SE-IgE, and IL-5) that are increased in nasal polyps tissue when there is comorbidity (asthma). Different microbiota might represent different functional communities and invoke an immune response ranging from homeostatic to detrimental inflammatory effects, as such explaining the differences between the different endotypes.

Aberrant bacterial community in CRSwNP disease.

This study identifies that the relative abundance of *H. influenzae* is remarkably higher in CRSwNP cases than in normal subjects. Combining the experimental data from mice [31], the epidemiological data from humans [32], and our observation of an association of *H. influenzae* with CRSwNP, we hypothesize that *H. influenzae* can initiate and drive inflammatory responses to develop nasal polyposis and therefore it may be hypothesized that elimination of *H. influenzae* in an acute phase might reduce incidence of NP cases. It might further be assumed that success or failure of the antibiotic treatment of the acute phase infection might drive an ongoing inflammation into resolution or NP formation, respectively.

In addition, the bacterial community in CRSwNP+A patients showed dominance of the phylum Proteobacteria, caused by unexpectedly high numbers of *E. coli* (*Enterobacteriaceae*), although not significantly different from the other two groups, whereas *S. aureus* was predominant in CRSwNP-A patients. Our findings concurred with a previous study that reported strong increase of *Enterobacteriaceae* in bronchial lavage of patients with asthma compared to those of control subjects [33]. When we combined the data of members of the Proteobacteria (i.e. *E. coli*, *H. influenzae*, and *M. catarrhalis*), we could establish a significant increase in relative abundance of these bacteria for patients with CRSwNP+A compared to those of the control group (Fig.S1b).

Proteobacteria might contribute to the pathogenesis of CRSwNP disease via eicosanoids and related mediators [34]. The abundance of *E. coli* in several CRSwNP+A patients and the positive correlation of *E. coli* with ECP and IL-5 suggest a role of *E.coli* in severity of type 2 inflammation in CRSwNP patients.

It is possible that *E. coli* impairs the epithelial barrier and cooperates with putative pathogens for initiation and amplification of type 2 inflammation in CRSwNP disease.

Effect of microbial components on putative pathogens

In the current study, the average relative abundance of *S. aureus* in CRSwNP-A patients is higher than that in healthy control subjects, but this is not the case for CRSwNP+A patients. The presence of specific IgE to staphylococcal enterotoxins (SE-IgE) is significantly more frequent in the CRSwNP+A than in the CRSwNP-A and control groups and correlates with disease severity. This observation supports the role of *S. aureus* and its immune proteome in the persistence of airway disease [35,36]. Apparently, our findings seem to indicate that the composition of the bacterial community may influence the *S. aureus* virulence. The presence of large numbers of Actinobacteria (e.g. *Corynebacterium spp.*) in the environment might shift *S. aureus* to benign colonization, whereas increased numbers of Proteobacteria (predominantly *E. coli*) in the milieu might drive *S. aureus* to exhibit pathogenic behavior. It may be conceivable that bacterial composition and interactions in the community culminate in a functional community that organizes commensal-pathogen interchange. Intraspecific variability cannot be ruled out, whereby *S. aureus* strains present in healthy samples could differ from strains from samples from diseases patients. In one study, a strain of *S. aureus* inhibited other strains of the same species co-colonizing in nasal cavity [37]. Various hypotheses were proposed to determine the regulation of *S. aureus* virulence, of which the global control of accessory gene regulator (*agr*) is the most promising one [38]. We also identified strain-level variations of *S. aureus*, classified by *agr*, in an independent survey and *in-vitro* bacterial interaction experiments. *S. aureus* RN 6390 belonging to *agr* group I and *S. aureus* ATCC 29213 *agr* group II. *S. aureus agr* group I had a positive impact on *C. pseudodiptheriticum*, whereas *S. aureus agr* group II had a negative impact on *C. pseudodiptheriticum*. Moreover, *S. aureus agr* group II killed

P. acnes in our experiments. A survey of *S. aureus agr* in higher numbers of patients should be conducted to confirm the possible importance of *agr* diversity of *S. aureus* in pathogenesis of CRS diseases.

Corynebacterium species have been implicated as competitors with *S. aureus* in the nasal niche [39,40] or as tempering *S. aureus* virulence [41]. One research group observed that *C. pseudodiphtheriticum* was present more often in non-*S. aureus* carriers than in *S. aureus* carriers [42]. This group confirmed a competitive interaction between *S. aureus* and *C. pseudodiphtheriticum* [42], which is not supported by our data. We detected *C. pseudodiptheriticum* more frequently in the CRSwNP status. In addition, our *in-vitro* interactions rather indicated a positive cooperation between *C. pseudodiptheriticum* and putative pathogens, such as *E. coli*, *H. influenzae* and *S. aureus (agr* group I).

Although several studies have described a negative association between *S. aureus* and *S. epidermidis* in the nasal cavity [43,44], our data did not show competition between *S. aureus* and *S. epidermidis*.

Bacterial Associations with Inflammation in CRS

A study using only 7 samples from CRS patients and a not-validated mice model concluded that *Corynebacterium tuberculostearicum* was a pathogen of CRS [45], while another study reported that CRS patients with enriched *C. tuberculostearicum* colonization in their nose, at the time of endoscopic sinus surgery, showed better surgical outcomes [10]. However, in our study neither a beneficial nor pathogenic role of *C. tuberculostearicum* can be concluded. We found that the distribution and relative abundance of *C. tuberculostearicum* was equivalent among groups. We also observed a negative correlation between eosinophilic activity in the tissue (i.e., ECP and IL-5) and other members of genus *Corynebacterium* (i.e., *C. macginleyi*, and *C. accolens*) that belong to phylum Actinobacteria. According to our previous study, the level of IL-5 is one of the prognostic factors in CRSwNP disease [14]. These results suggest that members of phylum Actinobacteria, i.e.,*Corynebacterium spp*. (e.g. *C. macginleyi*), are associated with a lower degree of eosinophilic inflammation in CRSwNP patients.

Health-associated Microbes

Several bacterial phyla were dominant in healthy individuals. The potentially beneficial microbe in the current study was a member of the phylum Actinobacteria; we found that *Propionibacterium acnes* was the most abundant microorganism in the healthy condition. Normally, *P. acnes* is a commensal microbe of adult human skin [46]. *P. acnes*, which prefer a lipid-rich anaerobic environment, has been detected in sinonasal cavities of healthy adults [9,42,44,47]. *P. acnes* has been described to promote Th1 and Treg cells and relieves atopic dermatitis symptoms [48], possibly by the production of short-chain fatty acids that are immunomodulating. Moreover, the principal metabolite of *P. acnes*, propionic acid, not only reduces inflammation by effects on macrophages [49], but also inhibits *S. aureus* growth [50]. This argument nevertheless remained unsupported by our pilot study. The levels of propionic acid measured in the sinonasal environment did not reach the minimum inhibitory concentration of *S. aureus*. Furthermore, *P. acnes* did not prevent the growth of *S. aureus*, which in turn killed *P. acnes* to establish its own growth. These data do indicate that *P. acnes* is an commensal rather than a defender, although *in-vitro* data regarding to bacterial interactions cannot be straightforwardly transposed to the *in-vivo* situation. *P. acnes* probably possess other factors for its fitness in healthy subjects. Healthy hosts may provide nutrients for *P. acnes* existence. Health-associated microbes should be further investigated to identify a genuine probiotic for the treatment of CRS disease.

This study provides a blueprint for identifying important microbial influencers of disease and provides information on the structure and members of nasal microbiota linked to specific clinical phenotypes and endotypes of CRSwNP disease (Fig.12). Our findings highlight the need for more functional studies on the bacterial community to determine the role of microbiota in CRS pathogenesis.

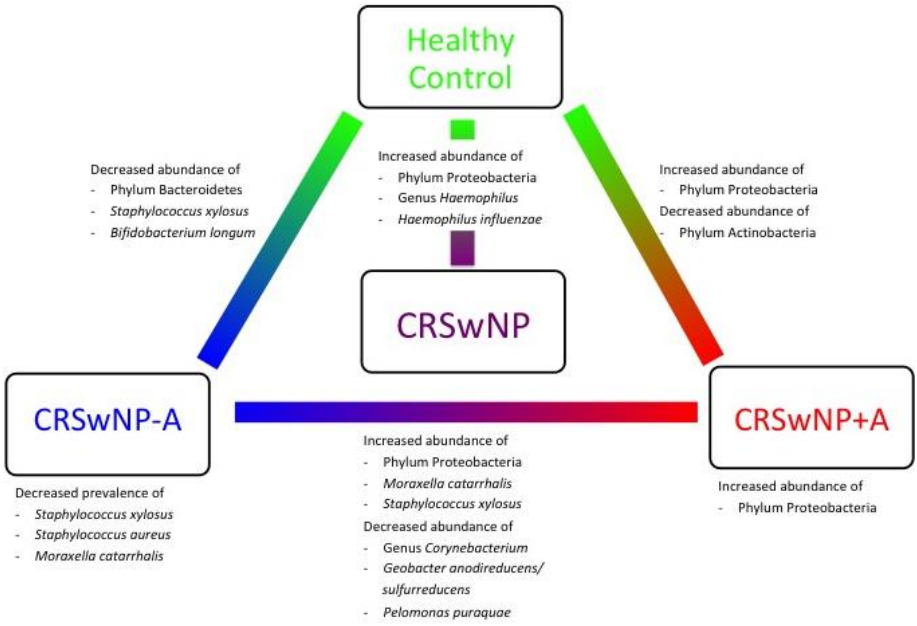

Figure 12. Aberrant bacterial community among groups.

Limitations

Several limitations should be addressed. This study was conducted using a limited number of samples, coming from patients who had prolonged medical treatment. Although we excluded patients that used steroids systemically, all CRSwNP patients may have taken intranasal steroid until shortly before the biopsy. The effect of steroids on bacterial communities remains unclear, although nasal irrigation with budesonide did not change the proportional abundance of bacteria in CRSwNP patients [51]. We were concerned about the specificity of molecular techniques, even though it has been shown that the relative abundance of bacterial DNA had a positive correlation with colony-forming unit counts (CFUs) [30]. Comparing recovered DNA to live colony counting, it appeared that in diseased subjects, *Staphylococcus* DNA mostly came from living bacteria, whereas in healthy subjects, *Staphylococcus* DNA was mostly recovered from dead bacteria [52]. The method used here for microbiota may detect genetic material of non-viable microorganisms that distort the genuine microbial community composition.

Moreover, one bacterial cell carries from 1 up to 15 copies 16S rRNA gene copies per genome [53]. The analysis of 16S rRNA can provide only the information of bacterial presence, but not metabolically active microorganism. The extraction and analysis of metagenomic mRNA (the metatranscriptome) or proteomic or metabolomic research should be further performed to assess the functional and metabolic diversity of microbial communities. Interplays between bacteria in our experiments are performed under *in-vitro* conditions. Although the tested strains were not directly isolated from the studied subjects, the selected strains were relevant to the situation in the human nose. We tested the interaction of only two species of bacteria. Interactions among the full spectrum of bacteria *in-situ* conditions may be different. The participation of the third, forth, and more species probably affects those bacterial behavior differently. A complementarily ecological theory would need to explain overarching interactions and patterns for the microbiota. Future research should explore functional parts of bacterial community and their impacts on the nasal mucosa. Investigations of bacterial communities and their impacts on sinonasal immune responses may pave the way for a better understanding of how the microbiota contribute to the pathogenesis of CRS.

Conclusions

This study identifies a difference in nasal microbiota between healthy subjects and phenotypes of CRSwNP patients. Proteobacteria (such as *H. influenzae, E.coli, M. catarrhalis*) were associated with CRSwNP disease, especially with CRSwNP+A cases, whereas Actinobacteria (such as *P. acnes*, *Corynebacterium spp*.) were prevalent in the healthy status. However, *P. acnes*, a resident commensal bacterium in healthy subjects, do not play the protective role against *S. aureus* one could expect from its abundance. Furthermore, although *S. aureus* was found abundant in CRSwNP-A, but not in CRSwNP+A, the presence of SE-IgE in the latter group representing its immunologic fingerprint rather than *S. aureus* itself is associated with disease severity. The functions of microbiota and their products in health and airway disease should be further elucidated to determine the effective role of microbiota in the pathomechanism of CRS disease.

Supplementary information

Taxonomy of relevant bacteria in the study

Phylum	Genus	Species
Proteobacteria	*Haemophilus*	*Haemophilus influenzae*
	Escherichia	*Escherichia coli*
	Moraxella	*Moraxella catarrhalis*
	Geobacter	*Geobacter anodireducens/sulfurrreducens*
	Pelomonas	*Pelomonas puraquae*
Firmicutes	*Staphylococcus*	*Staphylococcus aureus*
		Staphylococcus xylosus
	Streptococcus	*Streptococcus pneumoniae*
Actinobacteria	*Bifidobacterium*	*Bifidobacterium longum*
	Propionibacterium	*Propionibacterium acnes*
	Corynebacterium	*Corynebacterium pseudodiphtheriticum* *Corynebacterium macginleyi* *Corynebacterium accolens*

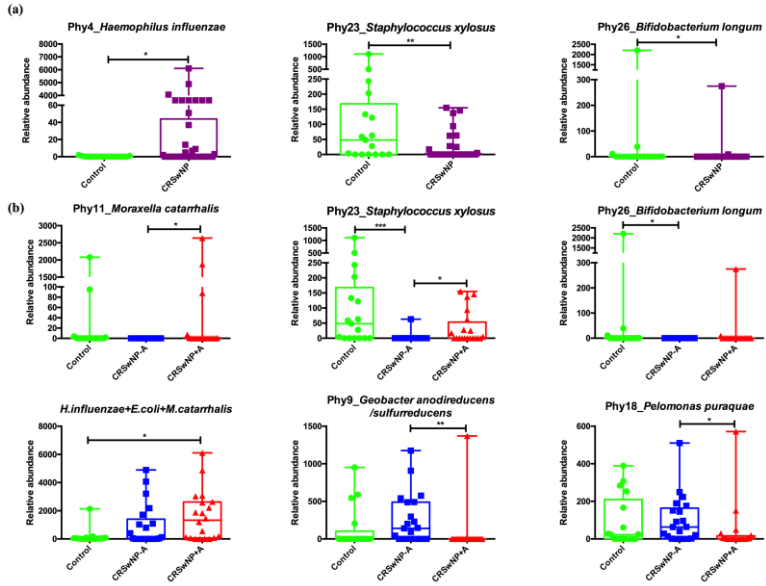

Figure S1. Bacterial species that discriminate between groups (a) and among groups (b).

The graphs show the relative abundance of discriminative species different between groups. Dots represent relative abundance in each sample.

Legend: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

Top growth (spots on top of inoculated plate)

Bottom growth (inoculated plate)	S. aureus ATCC29213 agr group 2	S. aureus gfp RN6390 agr group 1	E. coli O157:H7	C. pseudodiphtheriticum CIP 103420T	H. Influenzae ATCC49144	P. acnes ATCC6919
S. aureus ATCC29213 on chocolate agar in 37°C and 5% CO_2	= N	- N	= N	0 N	0 N	0 N
S. aureus gfp RN6390 on chocolate agar in 37°C and 5% CO_2	= N	= N	= N	+ N	= N	0 N
E. Coli O157:H7 on chocolate agar in 37°C and 5% CO_2	= N	- N	= N	- N	0 N	0 N
C. pseudodiphtheriticum on chocolate agar in 37°C and 5% CO_2	= N	= N	= I	= N	= N	= N
H. Influenzae ATCC49144 on chocolate agar in 37°C and 5% CO_2	= N	= N	= N	+ N	= N	0 N
P. acnes ATCC6919 on chocolate agar in 37°C and 5% CO_2	= D	= N	= N	= N	= N	= N

= = no different growth of inoculated point when compared with control plate

\+ = more growth of inoculated point when compared with control plate

− = less growth of inoculated point when compared with control plate

0 = no growth of inoculated point

N = normal growth of inoculated strain

I = increased growth of inoculated strain around inoculated point

D = decreased growth of inoculated strain around inoculated point

agr = accessory gene regulator

Figure S2. Mutual inhibition and growth promotion between different bacterial species that are most abundant in controls and patient groups.

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Chapter 8.
Discussion

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Rhinosinusitis, an umbrella term for various kinds of inflammatory diseases of the nasal and paranasal cavities, is characterized by two or more symptoms. One is either blocked nose or running nose. The other one could be facial pain or pressure, reduction or loss of smell, or both. The illness gains a chronic status when the symptoms persist for three months without recovery despite being treated [1]. Chronic rhinosinusitis (CRS) affects approximately 10% of the population in the United States and European countries [2,3]. This malady can be classified into two clinical subgroups (phenotypes) based on the absence or presence of nasal polyps (CRSsNP and CRSwNP respectively) [1]. This classification does not provide full insight into all underlying cellular and molecular pathophysiologic mechanisms. CRS is now recognized as various kinds of inflammatory diseases of the nasal and paranasal cavities [4]. The differentiation of the inflammation types based on T helper cells or biomarkers allows a more differentiated classification. We can classify according to pathomechanical principles into so-called endotypes within the clinical phenotypes [5,6]. Generally inflammation of CRSsNP is mostly Th1-skewed neutrophilic inflammation with elevated levels of interferon-γ (IFNγ) and transforming growth factor-β (TGFβ), whereas inflammation in CRSwNP is often Th2-skewed eosinophilic inflammation with elevated levels of IL5 and IgE [7]. Patients with CRS also form a heterogeneous group with different endotypes, meaning that inflammation patterns and T helper subsets vary. A well-defined endotype of CRS disease can identify specific pathological or immunological characteristics of the inflammation. The inflammatory pattern can be related to the type of bacterial colonization.

Association of microbes with CRS

In chapter 6, we showed the association of mucosal organisms with patterns of inflammation in CRS. With the Uniprobe, we detected bacteria in healthy control tissues. The intramucosal presence of bacteria has been demonstrated not only using molecular methods, but also using culture techniques [8]. Apparently, the sinus mucosa is not sterile and bacteria can colonize inside the tissue without

triggering inflammation. This finding supports the relationship between host and microbes in a healthy state. Although bacteria were detected in control tissues, the putative pathogens were significantly more present in diseased tissues than in control tissues. *S. aureus* was associated with IL-5 and SE-IgE positive sinonasal tissues of CRSwNP patients. A previous study showed that the presence of IL-5 and SE-IgE in sinonasal tissue is associated with comorbid asthma [9]. Contrary to our expectations, intramucosal presence did not correlate with bacterial load on the mucosal surface. The abundance of *S. aureus* of CRSwNP with comorbid asthma group was similar to those of control group. *S. aureus* may behave in a commensal or pathogenic fashion based on strain, bacterial gene expression, environmental conditions, and possibly based on surrounding microbial interactions. We found variations of *S. aureus* strains according to the *agr* system between the healthy control group and CRSwNP group. The majority of *S. aureus* strains isolated from CRSwNP patients expressed *agr* group I and *agr* group II. According to our findings, *S. aureus agr* group I and group II strains are more virulent than *S. aureus agr* group III and group IV strains. The role of *S. aureus* in sinonasal inflammation has been shown to go beyond the traditional model of infectious inflammation. *S. aureus* can reside in sinonasal epithelium and release enterotoxins that may contribute to the Th-2 response in CRSwNP context. In CRSsNP disease, the mucosal presence of *P. aeruginosa* was significant in sinus tissue of CRSsNP patients with TNFα > 20 pg/mL. According to the visual analog score, CRSsNP patients who have TNFα > 20 pg/mL in their sinus tissue have remarkably more pain symptoms than CRSsNP patients who have TNFα < 20 pg/mL in their tissue. In addition, the sinus tissues with TNFα > 20 pg/mL coincide with an elevated level of IFNγ and IL-22. From these findings we can presume that *P. aeruginosa* contributes to the pathogenesis of Th1 and/or Th22 CRS disease. Elimination of *P. aeruginosa* possibly reduces the inflammation and pain symptoms in CRSsNP patient. We found variations of intramucosal bacteria among the different CRS endotypes. Our findings reveal that *P. aeruginosa* is associated with Th1 and/or Th22 CRS disease whereas *S. aureus* is associated with Th2-bias CRS disease. These findings indicate that bacteria probably have an impact on the type of CRS inflammation.

Disruptions of the Microbial Composition in CRSwNP.

In chapter 7, the sinonasal microbiota of CRSwNP group showed significantly decreased bacterial diversity and evenness when compared with the healthy control group. These findings indicate the higher relative abundance of a few species in the bacterial community of in CRSwNP patients than those of healthy subjects. Further analysis revealed that *Haemophilus influenzae* was significantly more abundant in CRSwNP patients than in healthy control subjects. *H. influenzae* is the main causative infectious bacteria implicated in acute rhinosinusitis [10]. *H. influenzae* produces toxins that interfere with ciliary function and that damage mucosal cells [11]. *In vitro* experiments using human lung epithelial cell lines showed that *H. influenzae* penetrated into the subepithelial layer by passing between epithelial cells [12]. *H. influenzae* can invade and survive in a variety of human epithelial cells and macrophages. Intracellular *H. influenzae* was detected in the adenoidal cells [13], bronchial epithelial cells [14], and macropharge cells [15]. However, the intracellular colonization of *H. influenzae* in sinonasal epithelial cells is still unknown. Intracellular *H. influenzae* can avoid immune clearance mechanisms and cause a persistent infection. Persistent infection with *H. influenzae* can alter the adaptive immunity from Th1 to Th2 responses [16]. In a murine model *H. influenzae* was shown to orchestrate the immune system to aggravate allergic airway disease by decreasing regulatory T cell (Treg)-associated fork head box P3 (FOXP3) gene expression in lung tissues and increasing secretion of inflammatory cytokines IL-5 and IL-13 [17]. Interestingly, in humans, a deficit in $Foxp3^{+}$Treg cells was shown to be a characteristic of CRSwNP disease [18]. This evidence indicates that *H. influenzae* probably drives inflammatory responses in the development of nasal polyposis. Further investigation is needed to identify the role of *H. influenzae* in the pathogenesis of nasal polyposis.

Bacterial Communities and Impact on Inflammatory Pattern

In a subset of CRSwNP patients, the abundance of *S. aureus* between non-asthmatic patients and asthmatic patients did not reach the level of statistical significance. Furthermore, the *S. aureus* strain classified by *agr* group did not differ significantly between CRSwNP-A and CRSwNP+A groups. These findings indicate that other factors contribute to inflammatory patterns. CRSwNP patients with co-morbid asthma normally have a high level of IgE, SE-IgE, and IL-5 in their sinonasal tissues [6]. These biomarkers can be produced locally in the sinonasal mucosa, so resident microbes should contribute to the initiation and amplification of this reaction. Based on our finding, we can hypothesize that a Proteobacteria-rich condition probably aggravates type 2 inflammation in CRSwNP patients. Local microbes may induce mucosal inflammation under certain conditions. Local immunity may be regulated by either resident microbiota or microbial metabolites/components. Diseases that originate on mucosal membranes, like CRS diseases, might ensue from the action of polymicrobial communities of indigenous organisms, working in concert, resulting in the disruption of homeostatic mechanisms.

Conclusions and Perspectives

CRS is not a singular disease. Complex pathophysiology has been described in different CRS subgroups [6]. Sinonasal mucosa is a complex and dynamic ecosystem, which is inhabited by many microorganisms. Mucosal immunity is entrusted with the formidable task of protecting the host from microbial invasion while maintaining a peaceful coexistence with its resident microbiota. Changes in the composition of the nasal microbiota potentially impact the complex pathophysiology of CRS disease. The inflammations of CRS disease do not ensue from individual bacterium, but probably are resulted from an entire microbial community under the influence of specific conditions. The functional part of a microbial community should be investigated to determine the role of microbiota in pathogenesis of CRS disease. The function of bacterial communities probably forms the pattern that maintains physiological processes or initiates detrimental

inflammation of the host. The host-microbe interaction studies have involved introducing infection by individual organisms (and evaluating subsequent inflammatory responses), rather than infection by a collection of organisms simultaneously, which could conceivably yield different findings. In the future researchers should consider a model of studying human-microbe interaction by infecting with series of microbes. The role of nonbacterial microbiota (fungi, viruses) still remains to be investigated for their contribution to CRS pathogenesis.

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Summary / Samenvatting

Summary

Chronic rhinosinusitis (CRS) is a common disease that is characterized by prolonged mucosal inflammation of the nose and paranasal sinuses. CRS can be classified into two clinical subgroups based on the absence or presence of nasal polyps (CRS without (sine) nasal polyps, CRSsNP; or CRS with nasal polyps, CRSwNP). This classification suggests an individual therapeutic approach for patients. However, these broad phenotypes do not provide full insight into the potential underlying cellular and molecular mechanisms of CRS. Recognition of the heterogeneity of CRS has promoted the concept that CRS consists of multiple groups of biological subtypes (endotypes) that are defined by distinct pathophysiologic mechanisms. Multiple host and environmental factors have been implicated in the development of CRS; now more importance is placed on the role of microbes. The simple concept of a host interacting with a single pathogen has been replaced by a more complex combination of relationships between microbial communities and the host.

This study assesses the association between the upper airway microbiota and the severity of chronic rhinosinusitis. It is hypothesized that infectious processes are associated with inflammatory patterns. The most likely infectious agents in the context of persistent inflammation of sinonasal mucosa are bacteria. Previous research indicated that IL-5 positive nasal polyps are associated with Gram-positive bacterial colonization. *Staphylococcus aureus* significantly increases in a subgroup of chronic rhinosinusitis with nasal polyps, however various other microbes may play a role either in the progression or the regression of sinonasal disease. We employed peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) to detect intramucosal sinonasal tissue microorganisms. We furthermore used high throughput sequencing to detect the bacterial 16S ribosomal ribonucleic acids (rRNA) gene on the nasal mucosal surface. Relevant proteins were measured in sinonasal tissues to define the endotypes of CRS using ELISA, Luminex, and UniCAP systems.

Our findings suggest that intra-mucosal *S. aureus* is associated with Th2-bias CRSwNP tissues and sinonasal tissues from cystic fibrosis disease. In addition, sinonasal tissues of CRSsNP patients with TNFα > 20 pg/ml are associated with the intra-mucosal presence of *Pseudomonas aeruginosa*. Fungi are rarely detected in intra-mucosal tissue using the pan fungal probe, so it seems they do not play a major role in pathogenesis of CRS.

We found variations of bacteria on the surface of nasal mucosa in CRSwNP patients characterized by co-morbid asthma. *Haemophilus influenzae* was significantly enriched in CRSwNP patients when compared to healthy control subjects. The most dominant microbe was *Propionibacterium acnes* in healthy control subjects, *S. aureus* in CRSwNP patients without asthma and *Escherichia coli* in asthmatic CRSwNP patients. Furthermore, the most abundant bacterial species in the healthy state (i.e. *P. acnes*) could not inhibit survival or growth of the relevant bacterial species in CRS disease state (i.e. *H. influenzae, E. coli and S. aureus*). The levels of propionic acid measured in the sinonasal environment did not reach the minimum inhibitory concentration for a putative pathogen (i.e. *S. aureus*). These findings prompt us to further investigate the role of bacterial communities and products in pathophysiologic processes of chronic rhinosinusitis. We conclude that dysbacteriosis of the nasal mucosa is linked to inflammatory patterns in chronic rhinosinusitis.

Future research should use integrated approaches to link microbial structure and activity. Non-bacterial microbes (i.e. fungi and viruses) may also contribute to the community of interacting biological entities. The composition and function of the microbiota possibly play a significant role in disease pathophysiology. The interaction of microbes with mucosal immune responses and interspecies interactions within the sinonasal community warrants further investigation.

Samenvatting

Chronische rhinosinusitis (CRS) is een veelvoorkomende ziekte die gekenmerkt wordt door een langdurige ontsteking van het neusslijmvlies en de neusbijholten. CRS wordt ingedeeld in twee klinische subgroepen op basis van de af- of aanwezigheid van neuspoliepen (CRSsNP, CRS zonder (sine) neuspoliepen of CRSwNP, CRS met neuspoliepen). Deze classificatie suggereert een geïndividualiseerde behandeling van patiënten. Deze brede fenotypes bieden echter geen volledig inzicht in de mogelijke onderliggende cellulaire en moleculaire mechanismen van CRS. Erkenning van de heterogeniteit van CRS heeft het concept gepromoot dat CRS uit verschillende biologische subtypes (endotypes) bestaat, die worden gedefinieerd door verschillende pathofysiologische mechanismen. Meerdere gastheer- en omgevingsfactoren hebben een rol gespeeld bij de ontwikkeling van CRS; nu wordt er meer belang gehecht aan de rol van microben. Het eenvoudige concept van een gastheer die interactie heeft met één pathogeen is vervangen door een complexere combinatie van relaties tussen microbiële gemeenschappen en de gastheer.

Deze studie evalueert de associatie tussen de microbiota in de bovenste luchtwegen en de ernst van chronische rhinosinusitis. Men veronderstelt dat infectieuze processen geassocieerd zijn met ontstekingspatronen. De meest waarschijnlijke infectieuze agentia in de context van aanhoudende ontsteking van het sinonasaal slijmvlies zijn bacteriën. Eerder onderzoek wees uit dat IL-5 positieve neuspoliepen geassocieerd zijn met grampositieve bacteriële kolonisatie. *Staphylococcus aureus* neemt significant toe in een subgroep van chronische rhinosinusitis met neuspoliepen, maar verschillende andere microben kunnen ook een rol spelen bij de progressie of de regressie van rhinosinusitis. We gebruikten peptide nucleïnezuur fluorescentie in situ hybridisatie (PNA-FISH) om intramucosale sinonasale weefselmicro-organismen te detecteren. Verder gebruikten we “high-throughput sequencing” om de genen van de bacteriële 16S-ribosomale ribonucleïnezuren (rRNA) op het neusslijmvliesoppervlak te detecteren. Om de endotypes van CRS te definiëren, werden relevante eiwitten gemeten in sinonasale weefsels met behulp van ELISA, Luminex en het UniCAP systeem.

Onze bevindingen suggereren dat intra-mucosale *S. aureus* wordt geassocieerd met CRSwNP weefsels met een Th2-respons en met sinonasale weefsels van cystische fibrose. Bovendien zijn sinonasale weefsels van CRSsNP patiënten met TNFα > 20 pg/ml geassocieerd met de intra-mucosale aanwezigheid van *Pseudomonas aeruginosa*. Schimmels werden zelden gedetecteerd in intra-mucosaal weefsel met behulp van de "pan fungal probe". Het lijkt er dus op dat ze geen belangrijke rol spelen in de pathogenese van CRS.

Bij CRSwNP patiënten met asthmacomorbiditeit vonden we verschillende bacteriën op het oppervlak van het neusslijmvlies. *Haemophilus influenzae* was significant verrijkt bij CRSwNP patiënten in vergelijking met gezonde controlepersonen. De meest dominante microbe was *Propionibacterium acnes* bij gezonde controlepersonen, *S. aureus* bij CRSwNP patiënten zonder astma en *Escherichia coli* bij astmatische CRSwNP patiënten. Bovendien kon de meest overheersende bacteriesoort in de gezonde toestand (i.e. *P. acnes*) de overleving of groei van de relevante bacteriesoorten in de CRS toestand (i.e. *H. influenzae*, *E. coli* en *S. aureus*) niet inhiberen. De hoeveelheden propionzuur gemeten in de sinonasale omgeving bereikten niet de minimale inhiberende concentratie voor een vermeend pathogeen (i.e. *S. aureus*). Deze bevindingen sporen ons aan om de rol van bacteriële gemeenschappen en hun producten in pathofysiologische processen van chronische rhinosinusitis verder te onderzoeken. We concluderen dat dysbacteriose van het neusslijmvlies gekoppeld is aan ontstekingspatronen bij chronische rhinosinusitis.

Toekomstig onderzoek zou geïntegreerde benaderingen moeten gebruiken om de microbiële structuur en activiteit te koppelen. Niet-bacteriële microben (bv. schimmels en virussen) kunnen ook bijdragen aan de gemeenschap van interagerende biologische entiteiten. De samenstelling en functie van de microbiota spelen mogelijk een belangrijke rol in de ziektepathofysiologie. De interactie van microben met mucosale immuunreacties en interacties tussen species binnen de sinonasale gemeenschap rechtvaardigt verder onderzoek.

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